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FURTHER STUDIES ON PROGNOSIS OF BREAST CARCINOMA.

H. J. G. BLOOM.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W.1.

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In a recent communication in this Journal (Bloom, 1950) the significance of the histological architecture of the tumour in cases of mammary carcinoma was investigated. By employing a simple system of grading a close relationship was revealed between histology and prognosis.

A classification based upon the microscopic appearance of the tumour (grade) combined with a knowledge of its extent (stage) was found to give a far more accurate grouping of cases, and also provide a more reliable guide to prognosis than could be achieved by either a system of grading or staging alone. Furthermore, by considering the cases according to our clinico-pathological subdivisions, light was thrown upon some hitherto unexplained problems concerning the results of treatment.

The present paper is a continuation of the above work. It is the object now to investigate some further aspects of prognosis in breast cancer on which, from time to time, widely differing opinions have been expressed. What are these problems, and why have they given rise to confusion ?

Is the prognosis in carcinoma of the breast influenced by the age of the patient ? What is the effect on prognosis of delay in seeking treatment ? Has the size and also the site of the primary growth any bearing on outcome ? These questions will be studied presently, and the significance of the histological grade of malignancy of the tumour will be considered in relation to them.

Material.

The same series of cases employed in our first communication will be used for the present enquiry. It is made up of 565 cases of breast cancer treated at the Middlesex Hospital during the years 1936 to 1942, and at the associated War Sector Units during the latter half of this period. From these patients 95 must be excluded for various reasons already considered (Bloom, 1950) thus leaving us with 470 available for the investigation.

Method of Grading.

The system of histological grading introduced into this country by Patey and Scarff (1928) will be used. This is based upon the principles formulated by

Greenough (1925) in America, but chief importance is attached to the degree of tubule formation, regularity in size and staining of nuclei, and the number of hyperchromatic and mitotic figures. Three classes of tumour are recognized and designated Grade I, Grade II and Grade III, thereby indicating carcinomata of low, intermediate and high degrees of malignancy, respectively. Further details of this method of grading may be found in our previous report (Bloom, 1950), the system proving both simple to apply and remarkably effective in determining possible outcome. The five-year survival rates for the three classes of tumour were as follows: Grade I, 79 per cent; Grade II, 42 per cent, and Grade III, 25 per cent.

Assessment of Prognosis.

The prognostic yard-stick will be the five-years' survival rate. It must be pointed out that " survival rate " does not necessarily imply freedom from cancer; it is merely an indication of the number of patients actually alive.

AGE AND PROGNOSIS.

Many authors consider age to have an important bearing on prognosis in cancer of the breast. Lee and Stubenbord (1928) have included a factor for age in their clinical classification which has been employed recently by Richards (1948).

It is widely believed that the younger the patient with malignant disease, the more gloomy the outlook. In the case of mammary carcinoma this view is held by MacCarty and Sistrunk (1922), Perry (1925), Daland (1927), Stout (1932), Nathanson and Welch (1936), Kunath (1940), Cade (1940) and Pack and Livingstone (1940).

Breast cancer occurring before the age of 30 is often regarded as being extremely fatal, and Haagansen and Stout (1943) point out that some surgeons go so far as to decline to operate upon such cases. Lee (1931) made a special study of this disease in young women. He investigated 191 cases treated by radical surgery with or without ancillary radiotherapy; all were under the age of 40. The three-year survival rate was 40 per cent, and only 16 per cent were without obvious metastases. The author concluded that " cancer of the breast in young women is a much more menacing disease than it is in mid-life or in old age." It is true that these results may be poor for such a short period of follow-up, but attention must be drawn to the fact that no control series of older patients treated at the same clinic was available for comparison. In addition, when the survival rate was determined for various age groups under 40 the results were found to be practically identical. Furthermore, there were 25 cases of lactation cancer in the series, and these should have been considered separately as they represent a special, well-recognized problem. The effects of pregnancy or lactation on breast cancer will be referred to later.

At the Middlesex Hospital, Lazarus-Barlow and Leeming (1924) made their classical study on the natural duration of life for cancer of various organs. With regard to the breast, 243 cases, seen between the years 1883 and 1922, were available for study. The authors revealed a parallel between age and outcome, the mean duration of life becoming greater with advancing years.

We must now turn and consider the opposite view, that age is of no prognostic significance. de Cholnoky (1943) reported the findings in a series of cases,

all under the age of 30. Fifty-nine patients were treated by a radical mastectomy with a five-and ten-year survival rate of 41 per cent and 37 per cent respectively. These results are relatively good. In fact, they are even superior to those obtained in a general series studied by the same author—34 per cent five-year and 22 per cent ten-year survival rates (Eggers, de Cholnoky and Jessup, 1941).

Hawkins (1944), in an investigation of some 2,600 cases showed a comparable five-year survival rate in three age groups, namely, 49 and less, 50 to 59 and over 60. The author concluded that these findings do not support the frequent contention that the younger cancer patients have a worse outlook than the older groups.

Age was not found to be an important factor in prognosis by Wyard (1925), Greenwood (1926), Evans and Leucutia (1930), Lewis and Reinhoff (1932), Matthews (1933), Scarff and Handley (1938), Macdonald (1942), Hoopes and McGraw (1942), Haagensen and Stout (1943), Truscott (1947) and Harnett (1948).

Lane-Claypon (1924) in her survey for the Ministry of Health demonstrated a similar survival rate among some 500 cases classified according to decade. In a further paper (Lane-Claypon, 1928) some 1,800 cases were divided into three age groups—less than 40, 40 to 50, and 60 and over. From a study of the survivals for each group, the author concluded that " the statement commonly made that the prognosis is worse in younger persons is erroneous."

More recently, Harrington (1946) in a large series of cases from the Mayo Clinic found no relationship between age and the number of survivals at five years. In addition, the effect of age on the incidence of axillary metastases was investigated. The proportion of cases with this complication were found to be comparable in the majority of groups. Similarly, 59 per cent of the young patients reported by de Cholnoky (1943) had axillary involvement compared with 64 per cent in the same author's general series (Eggers, de Cholnoky and Jessup, 1941).

Can a relationship between age and prognosis be shown in the present series ? The age was available in 461 cases. The youngest patient was 22 and the oldest 80. Table I displays the five-year survivals in the various decades. The results of a broader grouping are also presented (Table II).

If we examine Table I it is evident that there is considerable fluctuation in the percentage of survivals in the different groups. No parallel is revealed

TABLE I.—*Age and Prognosis.*

Age.	Cases.	5-year survivals.	
		No.	Per cent.
<40	45	19	42
40–49	145	84	58
50–59	136	66	49
60–69	87	33	38
70+	48	24	50

between age and outcome, and there is no support for the view that the tumours of younger women, apart from those associated with pregnancy and lactation, kill more rapidly. Of patients aged 39 or less, 42 per cent were alive at five years compared with 38 per cent in the seventh decade. When the cases are separated out, as in Table II, it is clear again that the younger patients fare no worse than

do the older ones, in fact, if anything, they do rather better. This has also been the finding of Lane-Claypon (1928) and Hawkins (1944).

TABLE II.—*Age and Prognosis.*

Age.	Cases.	5-year survivals.	
		No.	Per cent.
<50	190	103	54
50–59	136	66	49
60+	135	57	42

Geschickter (1945) believes that certain unfavourable features exist for younger women with breast cancer, but that these are compensated for by the fact that such patients, as shown by Kaae (1948), tend to seek medical advice sooner than do older groups. Where the prognosis has been found to be comparable for various ages it may be argued that this factor has come into operation. Hence, it may not be adequate to consider the prognostic significance of age purely from the results of treatment.

Many authors who claim a parallel to exist between age and outcome maintain that the breast cancers of younger women tend to be more anaplastic and grow more rapidly than do those in older women. If this is true one would expect to find a greater proportion of growths of high grade malignancy among the younger cases, and a preponderance of low grade tumours in the older groups. Such a discovery would be conclusive evidence for the view that the younger the patient the graver the outlook. Now, several workers have shown that the degree of malignancy in mammary carcinoma can be ascertained by examining a section of the tumour. Attempts, however, to correlate the histology of cancer with age appear to have been rarely performed. Up to the present time only two investigations of this type have been found in the literature (Taylor, 1936 ; Lees and Park, 1949). A study on these lines will now be made.

TABLE III.—*Age, Grade and Prognosis.*

Age.	Grade.	Cases.	
		No.	Per cent.
<40	I	10	22·2
	II	22	
	III	13	28·9
40–49	I	45	31·0
	II	58	
	III	42	29·0
50–59	I	39	28·7
	II	59	
	III	38	27·9
60–69	I	26	29·9
	II	37	
	III	24	27·6
70+	I	16	33·3
	II	16	
	III	16	33·3

Table III has been constructed to show the relationship between age and grade of malignancy in breast cancer. An examination of this table reveals a really remarkably even distribution of tumours of low and high grade malignancy in the various decades. Not only is the proportion of Grade I and also of Grade III cases similar in the different groups, but in addition the incidence of these tumours is practically identical in the individual groups. Thus we can find no support whatsoever in the present study for the thesis that cancer of the breast is more malignant in younger than it is in older patients. These results, however, are not in agreement with those obtained by Taylor (1936) who also employed the principles of grading laid down by Greenough (1925). He found a smaller number of low and a greater number of high grade tumours among younger women when compared with an older group. On the other hand, the conclusions reached by Lees and Park (1949), who have recently investigated the relationship between histology and age for cancer in various organs including the breast, agree with our findings and not with those of Taylor (1936).

DURATION OF SYMPTOMS AND PROGNOSIS.

Generally speaking, the delay in seeking medical advice after the onset of symptoms in breast cancer is becoming shorter. Towards the end of the last century we find Dietrich (1892) reporting only 23 per cent of cases in a series as having symptoms lasting less than six months. Reports from many sources indicate that the proportion of patients attending hospital within this time has gradually increased over the course of the last fifty years. For example, according to Harrington (1946) 33 per cent of the cases seen at the Mayo Clinic between the years 1910 and 1914 were operated upon within six months and 54 per cent within one year of the first symptom. These percentages climbed steadily, reaching 50 and 70 respectively for the period 1935 to 1939.

In the present study data regarding the length of the history were available in 406 cases. Of these, 65 per cent came for treatment within six months of the first symptom and 85 per cent within one year. Similar percentages are given by Kaae (1948). In spite of the improvement, compared with earlier investigations, these figures are still far from satisfactory. On the other hand, reports from elsewhere are much more gloomy. Lewis and Reinhoff (1932) find as few as 34 per cent of their cases seeking medical advice within six months and 63 per cent within one year. Even more recently, Eggers, de Cholnoky and Jessup (1941) quote only 37 per cent and 55 per cent for corresponding intervals of time.

The symptoms of mammary carcinoma and their frequency are dealt with adequately by many authors. Suffice it here to say that by far the commonest initial symptom has always been a lump in the breast. Forty-five years ago Campiche and Lazarus-Barlow (1905) made a study of nearly 2,000 patients seen at the Middlesex Hospital between the years 1747 and 1903, and found this feature present in 63 per cent. Later, at the same hospital, Beckton (1909) gave an incidence of 70 per cent. More recent authors report even higher figures. Thus, Harnett (1948) and also Cade (1940) find a lump to be the first symptom in 77 per cent of cases, Truscott (1947) in 84 per cent and Putzki and Scully (1946) in 88 per cent. The " duration of symptoms," therefore, in the vast majority of cases refers to the length of time a tumour has been present in the breast—from the time the patient found it to the time she attended the hospital clinic.

The responsibility for prolonged delay before the institution of treatment may

be entirely that of the patient, but not always. Medical practitioners are occasionally guilty of wasting time, especially with regard to younger women when cancer is not suspected. Kaae (1948) found that in his series three-quarters of the cases were subjected to adequate therapy within one month and 90 per cent within six months of the first medical examination. Lane-Claypon (1926) reported a mean interval of five to six months between the consultation and operation among 670 cases.

Whenever the management of malignant disease is discussed the importance of early diagnosis and early treatment are emphasized. In the case of mammary carcinoma, has the duration of symptoms any bearing on prognosis ? The general teaching has certainly always been that the greater the delay in seeking treatment in this disease the worse the outlook. The impression is also frequently given that if a patient receives early therapy the prognosis is necessarily good. These views meet with almost universal acceptance, and only very rarely have they been questioned. Are they in point of fact well-founded ? For if they are, we should direct more attention to reducing the tragically large numbers of women who, through fear or ignorance, fail to seek medical care until many months have elapsed following the discovery of a lump in the breast. If we can accomplish this, will the number of survivals at five and ten years after operation increase ? Many authorities believe they will.

Let us now consider the opinions which have been expressed regarding the duration of symptoms and prognosis. Luff (1932), in an investigation of over 1,500 cases for the British Medical Association, found that the longer the delay between the first symptom and operation the more gloomy the outlook, as shown by the survival, recurrence and mortality rates (Table IV). A study of this table reveals that with a delay of twelve months the survival rate is halved and the recurrence rate more than doubled. Cade (1950) refers to Luff's work and states that " the mortality of cancer of the breast in England and Wales could be reduced from 7,000 yearly to 1,000, if all cases were adequately treated in the first month of the appearance of the disease."

TABLE IV.—*Prognosis and Duration of Symptoms* (*Luff*, 1932).

Delay in months.	4-year survival rate. Cases per cent.	Post-operative recurrence. Cases per cent.	Mortality rate. Cases per cent.
< 1	31	14	5
1–3	29	14	17
3–6	24	27	16
6–12	24	27	26
> 12	16	35	37

Davis (1938), after studying a small series of 75 cases, considers that one of the most important factors in determining prognosis is the delay in treatment. He states that early diagnosis and therapy are essential in the ' cure ' of cancer of the breast, and raises the question of advisability of surgery for patients with a long history.

Hoopes and McGraw (1942) found that the percentage of five-year survivors diminished slightly with increase in duration of symptoms, regardless of whether or not the axillary glands were invaded.

Macdonald (1942), in a large series of cases, revealed that the greatest proportion of five-year survivals were among those treated within two months of the tumour being discovered. After this period a rapid deterioration in results occurred. With a delay of one year, however, it was surprising to find the numbers alive increasing. Similar results were obtained by Eggers, de Cholnoky and Jessup (1941), who report a five-year survival rate of 76 per cent for patients operated upon within one month. Here again, the results deteriorated rapidly with increasing duration of symptoms, falling to 20 per cent for a delay of one to two years. With further loss of time, however, the results improved, from 25 per cent for a lapse of two to three years to 41 per cent for an even greater interval.

From the preceding data it would appear that duration of symptoms has a direct bearing on prognosis—the longer they have been present the worse the outlook. The significance of the increase in the survival rates after a delay of one or more years, as found by Macdonald (1942) and also by Eggers, de Cholnoky and Jessup (1941), will be discussed later.

What can be revealed in the present series of cases as to the relationship between delay in seeking treatment and prognosis ? Information regarding this point was available in 406 instances. The five-year survivals were determined according to the duration of symptoms, and these are displayed in Table V. It

TABLE V.—*Duration of Symptoms and Prognosis.*

Duration of symptoms.	Cases.	5-year survivals.	
		No.	Per cent.
6 weeks or less	101	50	50
3 months or less	184	94	51
3–6 months	125	59	47
6–12 months	118	56	47
12 months or more	105	55	52

is to be noted that some overlap occurs in the various groups. Examination of this table shows a truly remarkable state of affairs. The survival rate is uniform, no matter how long the history. We are not able, therefore, to agree with the findings of Luff (1932), Davis (1938), Eggers, de Cholnoky and Jessup (1941), Hoopes and McGraw (1942) and Macdonald (1942). Our results also appear to conflict with generally accepted views, but they are not unique. Lewis and Rienhoff (1932) in 950 cases, Hawkins (1944) in over 3,000 cases and Kunath (1940) consider that duration of symptoms, *per se*, are of little prognostic significance.

This problem must be now examined more closely. So far, we have studied the cases as a whole. No attempt has been made to classify them, and the importance of this procedure has been stressed elsewhere (Bloom, 1950). What types of cases are present in the various groups shown in Table V? It is possible that these groups are not comparable. For example, there may be a preponderance of highly malignant tumours among the women attending hospital within the six-week period, whilst there exists but a small proportion of similar growths among those delaying for one year or longer. Such a distribution may compensate for the difference in time, and thus account for the practically identical end-results.

Cases of breast cancer may be classified by clinical stage or histological grade.

Let us first of all consider the scatter of the patients according to stage and duration of symptoms. For this purpose the Manchester system was employed, a summary of which may be found in our previous report (Bloom, 1950). Although this classification depends upon clinical data, glandular involvement was determined, where possible, by microscopic examination; the reasons for this have been dealt with in the same communication.

Reference to Table VI shows that with increasing length of history there is a progressive fall in the proportion of cases at an early stage (Stage 1). A similar

TABLE VI.—*Distribution of cases according to Duration of Symptoms and Clinical Stage.*

Duration of symptoms.	Total cases.	Cases in Stage 1. Per cent.	Cases in Stage 2. Per cent.	Cases in Stage 3. Per cent.	Cases in Stage 4. Per cent.	Total. Per cent.
6 weeks or less	101	39	38	19	4	100
3 months or less	184	37	39	19	5	100
3 to 6 months	125	35	34	23	8	100
6 to 12 months	118	33	30	31	6	100
1 year or more	105	25	21	44	10	100

state of affairs is seen for those in Stage 2. On the other hand, the advanced cases (Stages 3 and 4) increase in number with prolonged delay. In other words, as more and more time elapses from the onset of the disease, as determined by the patient, so the early and moderately advanced tumours fall in number whilst the proportion of more extensive growths becomes greater.

These results deserve no special comment. They are what one expects and support the conclusions of Kaae (1948) that "with increasing duration of symptoms the number of Stage 1 cases becomes steadily less and there is a gradual increase in the number of inoperables. . . ." On the other hand, these findings make it even more difficult to understand those shown in Table V. For instance, the percentage of Stage 1 and Stage 3 cases among the patients presenting within six weeks of the first symptom is 39 per cent and 19 per cent respectively (Table VI). After a delay of one year or more the proportion of early cases falls to 25 per cent, whilst the advanced cases increase to 44 per cent. And yet, in spite of this, the five-year survival rate is practically the same—50 per cent for those having a delay of six weeks or less compared with 52 per cent for those waiting one year or longer (Table V).

Will a study of the histological type of tumour throw any light on the problem? The distribution of cases according to duration of symptoms and grade have been drawn out in Table VII. A comparable percentage of Grade I tumours is seen

TABLE VII.—*Distribution of Cases According to Duration of Symptoms and Histological Grade.*

Duration of symptoms.	Total cases.	Cases in Grade I. Per cent.	Cases in Grade II. Per cent.	Cases in Grade III. Per cent.	Total. Per cent.
6 weeks or less	101	24	45	31	100
3 months or less	184	25	45	30	100
3 to 6 months	125	23	47	30	100
6 to 12 months	118	33	40	27	100
1 year or more	105	38	34	28	100

among the patients attending hospital within six weeks, three months and three to six months of the first symptom. A similar state of affairs exists for Grade II and also Grade III carcinomas. After this time, however, there is an increase in the proportion of Grade I cases, and a reduction in those belonging to the higher grades of malignancy. These changes, although not marked, may indicate that the more virulent tumours tend to present sooner than the ones of lower malignancy.

Let us investigate this matter further by considering the delay in seeking treatment of the cases according to the grade of tumour. Table VIII has been

TABLE VIII.—*Percentage of Cases in Each Histological Grade According to Duration of Symptoms.*

Grade.	Duration of symptoms in months. 6 or less. Cases per cent.	7 to 12. Cases per cent.	More than 12. Cases per cent.	Total per cent.
I	56	21	23	100
II	70	18	12	100
III	69	21	10	100

constructed on a broader basis than Table VII, three periods only being taken, namely, less than six months, between seven and twelve months and more than one year. These results lend more weight to the view that patients with tumours of high malignancy tend to consult their doctors earlier than do those with growths of low malignancy. In addition, the mean duration of symptoms for the three classes of tumour are presented in Table IX. Here again there is evidence that

TABLE IX.—*Mean Duration of Symptoms According to Grade.*

Grade.	Cases.	Mean duration of symptoms in months.
I	124	10.1
II	174	7.6
III	125	7.1
Total	423	8.3

the most malignant neoplasms are associated with the shortest histories. This, presumably, depends upon the rate of growth of the cancer, the higher grades growing more rapidly. Thus, larger tumours are discovered more easily, and a rapid increase in size alarms the patient to seek medical advice at an early date. The small, slowly growing tumours of low grade malignancy may be regarded by patients, at first, as not being serious, and so more time elapses before the medical consultation.

Let us summarize briefly what has been said so far: Opposing views regarding the effect of delay in seeking treatment on prognosis have been considered. From our own series we have not been able to show, in spite of the findings in Table VI that outlook becomes more gloomy with increasing delay. It was only when the histological grade of malignancy was studied that some light was thrown on the problem. It appears that the distribution of the types of carcinoma compen-

sate to some extent for the time factor. With regard to the reports of other workers, it may well be that the conflicting results obtained depend upon the comparison of histologically incomparable groups of cases.

In view of these findings let us once again consider the relationship between duration of symptoms and prognosis, but this time according to the grade of malignancy (Table X). It is evident that outcome is now shown, in certain cases,

TABLE X.—*Duration of Symptoms and Prognosis According to Histological Grade.*

Duration of symptoms.	Grade I.		Grade II.		Grade III.	
	Cases.	Alive at 5 years. Per cent.	Cases.	Alive at 5 years. Per cent.	Cases.	Alive at 5 years. Per cent.
6 weeks or less	24	92	46	41	31	29
3 months or less	45	87	83	47	56	29
3 to 6 months	28	86	59	42	38	26
6 to 12 months	39	72	47	43	32	25
1 year or more	40	78	36	50	29	21

to be influenced by the length of history. Examination of this table in greater detail allows the following conclusions to be reached. The delay in seeking medical advice appears to be of some importance for Grade I cases; of these attending hospital within six weeks of the first symptom, 92 per cent survive five years, compared with 72 per cent waiting six to twelve months. In contrast, there is little difference in outlook for patients with Grade II tumours for similar intervals of time. In the case of the highest grade of carcinoma there is a negligible fall in results with increasing delay. The survival rate in this group is 29 per cent for patients seeking treatment within six weeks of the onset, compared with 25 per cent for those waiting six to twelve months. We are thus faced with the fact that by the time a highly malignant growth (Grade III) is discovered by the patient it is, in all probability, too late to eradicate, direct extension and metastasis having already taken place.

It must be pointed out, however, that many women with Grade III cancers who present early appear, from the clinical aspect, to have a favourable outlook. Why then is disaster so frequent in these cases? The theory is held here that such cases, in reality, are sub-clinically advanced, small tumour emboli probably having already reached regional glands and bone. Such deposits have not yet had time to grow and become evident. Even routine histological examination of axillary glands removed by the radical mastectomy may fail to reveal their presence. Saphir and Amromin (1948), by serial sections, have demonstrated cancer cells in the axillary glands in about one-third of cases previously reported as being free from invasion.

We are compelled to adopt the view that outcome in mammary carcinoma is determined largely by the histological type of growth, rather than by prompt treatment as soon as the lesion is discovered. Macdonald (1942) expresses a similar opinion when he states that "natural selection" is more important in prognosis than early therapy. Kunath (1940) considers that "the rate of growth" is the major factor influencing end-results rather than the length of time the tumour has been present. In fact, Nathanson and Welch (1936) believe that

patients with the shortest delay in treatment have the worst prognosis ! This unorthodox view is based upon evidence which suggests that patients with tumours of slow growth tend to seek medical aid much later than those with ones of rapid growth ; some support for this has been found in Tables VIII and IX.

Certain authors have shown a sudden increase in the five-year results when the duration of symptoms reaches one year or longer. This has been the experience of Eggers, de Cholnoky and Jessup (1941), Macdonald (1942) and Harnett (1948). In the present series no such increase was observed when the patients were taken as a whole (Table V). On the other hand, a slight improvement was demonstrated by considering the cases according to histological grade (Table X). This only applied to the carcinomas belonging to the low and intermediate grades of malignancy. It is postulated that these tumours of long duration were of particularly slow growth and, therefore, did not alarm the patients sufficiently to visit their doctors until after a considerable length of time had elapsed.

What conclusions can be reached here regarding the duration of symptoms and prognosis in breast cancer? Pleas are for ever being made for the education of the public and general practitioner with a view to shortening the delay in seeking treatment after the onset of the first symptom. This may appear to be justifiable when it is remembered that, even in modern communities subject to press and radio propaganda, there are still, at the best, only some 60 per cent of patients who present within six months of discovering a lump in the breast. Lost time, however, has been shown here to be of importance only for tumours of low grade malignancy. With regard to the most virulent growths (Grade III) this factor appears to be of little or no significance. The theory is put forward that by the time such a tumour is discovered by the patient it has already spread beyond the breast.

Perhaps we may now refer briefly to certain clinical implications arising from our investigation. What can be done to improve the prognosis of the Grade III cases ? It would appear that the favourable stage for the treatment of this type of growth is long before it is found by the patient. Frequent routine examinations of the breasts would, in all probability, lead to its early discovery, and perhaps a better chance for attaining a five-year survival. Several surgeons have suggested such a plan. Indeed, Hawkins (1944) has gone so far as to advocate that every woman should be taught to inspect and palpate her own breasts, and that such an examination be made not less than once a month. Chase (1947) voices a similar opinion. When it is recalled that about 7,000 women in this country and 15,000 in the United States of America die annually from breast cancer, some may consider this procedure to be warranted. Others, for fear of producing a widespread cancer neurosis, would rather direct energy to improving the treatment of the disease.

Doubt has been expressed as to the value of surgical treatment, purely on account of a long history (Davis, 1938). From the evidence presented in Table X it would appear that such a view is not only fallacious, but also extremely dangerous ; of the patients with symptoms lasting one year or more, over three-quarters of those belonging to Grade I and fully a half of those of Grade II were alive five years after operation.

In conclusion : we believe that campaigns aimed at shortening the delay before seeking medical advice should not only be continued, but also intensified, if only for the sake of those patients having tumours of low histological malignancy.

It is most important, however, to realize that such measures alone must not be relied upon to increase the number of five-and ten-year survivors. We have revealed that the prognosis of certain cases does not appear to be materially influenced by the institution of early therapy. In fact, it is misleading to state, as so often is done, that the outlook is usually good for patients who present promptly after the onset of symptoms.

SITE OF TUMOUR AND PROGNOSIS.

Before investigating the relationship between site of growth and prognosis in breast carcinoma, let us first examine the frequency with which various parts of the gland are involved by this disease.

The incidence of cancer in either breast appears to be about equal. A number of reports, however, suggest a slight preponderance, to the extent of 4 or 5 per cent, for the left side over the right (Luff, 1932 ; Wevill, 1932 ; Busk and Clemmesen, 1947 ; Harnett, 1948).

The region of the breast affected was studied in the present series, the usual subdivisions into quadrants, axillary tail and centre being made. Adequate information regarding the site of the growth was available in 441 cases. In 52 of these the tumour was described as being situated in a hemisphere, or in the midline of two adjacent quadrants. Four patients were said to have more than one lump in different quadrants of a single breast. It was thought best to exclude these 56 cases from the series, leaving 385 for consideration. The distribution of the tumours, which is drawn out in Table XI, reveals a well-marked preponderance of the upper outer quadrant of the breast as the site for mammary cancer.

TABLE XI.—*Incidence of Breast Carcinoma According to Site (Middlesex Hospital).*

Site.	Present series (1936 to 1942) 385 cases.		Campiche and Barlow, 1905 (1747 to 1903) 1010 cases.	Beckton, 1909 (1904 to 1909) 230 cases.	Truscott, 1947 (1926 to 1935) 836 cases.
	Cases.	Per cent.	Per cent.	Per cent.	Per cent.
Upper outer	177	46	45	46	46
Upper inner	75	19	17	20	20
Central	35	9	20	15	13
Lower outer	54	14	12	13	12
Lower inner	19	5	6	5	5
Axillary tail	7	2	–	1	4
Diffuse or whole breast	18	5	–	–	–
Total	385	100	100	100	100

The frequencies given here for the different regions agree very closely with those of previous workers at the Middlesex Hospital, also shown in Table XI. The somewhat higher incidence of " central " growths found by Campiche and Lazarus-Barlow (1905), Beckton (1909) and Truscott (1947) is probably accounted for by the inclusion in this group of large growths involving three or more quadrants. We have preferred to classify these separately under the heading of " diffuse " or " whole breast." The results obtained at this hospital differ in no

way from those reported elsewhere (Lane-Claypon, 1924; Luff, 1932; Wevill, 1932; Geschickter, 1945; Harnett, 1948).

Having noted the frequency with which the various parts of the breast are the seat of cancer, we will now consider whether these sites have any prognostic significance. Many opinions have been expressed, but there is no general agreement on this point. Handley (1922, 1927) was the first to stress the importance of the internal mammary chain of lymph glands as an avenue of extension for malignant cells to the thorax and abdomen. Because of the proximity to these surgically inaccessible nodes, carcinomas arising in the inner half of the breast are alleged by some authors, such as Bartlett (1933) and Hawkins (1944), to carry a more gloomy prognosis than those situated in the outer hemisphere.

It has also been stated that cancer in the upper outer or lower inner quadrants is more likely to prove fatal at an early date, the former being in close relation to the axillary lymph glands and the latter to the abdominal viscera. A similar opinion has been expressed for tumours in the region of the nipple, owing to the presence of the sub-areolar plexus of Sappey which drains to the axilla. Lane-Claypon (1924) found support for these views in a group of some 300 cases. This same author, however, in a later, more extensive study (Lane-Claypon, 1928) was unable to confirm her previous results, and finally decided that "there is no evidence that the prognosis varies according to the site." More recently, Truscott (1947) and also Harnett (1948) have reached a similar conclusion.

The relationship between site and survival rate was examined in the present series, the results being shown in Table XII. Owing to the very small number of

TABLE XII.—*Site and Prognosis.*

Site.	Cases.	5-year survivals.	
		No.	Per cent.
Upper outer	177	92	52
Lower outer	54	31	57
Central	35	15	43
Upper inner	75	35	47
Lower inner	19	9	47
Axillary tail	7	1	14
Diffuse or whole breast	18	3	17
Total	385	186	48

examples, we cannot consider the prognostic significance of axillary tail involvement. The cases classified as "diffuse" or "whole breast" appear to have a bad outlook, a mere 17 per cent surviving five years. As for the other sites, there is no striking variation in the results. It is possible that growths situated in the sternal quadrants and in the nipple area carry a slightly worse prognosis than do those in the outer regions.

A broader grouping of cases will now be considered, depending on whether the inner or outer hemisphere of the breast is the seat of the tumour (Table XIII).

TABLE XIII.—*Site and Prognosis.*

Site.	Cases.	5-year survivals.	
		No.	Per cent.
Inner hemisphere	94	44	47
Outer hemisphere	243	130	53

The survival rate is again seen to be less for the inner region, but the difference is very small and therefore of doubtful significance. Consequently, we have not been able, so far, to prove that tumours situated in the sternal half of the breast carry a more gloomy outlook than those in the axillary half. In fact, a previous writer from this Hospital (Truscott, 1947) found that patients with growths in the lower inner quadrant had the lowest mortality.

When we have been previously faced with conflicting opinions on various aspects of breast cancer, considerable assistance has been obtained by studying the morbid histology of our cases. Let us, therefore, apply the principle of grading to the present problem.

First of all, what types of cases are present in the various groups we have considered in Table XII ? The distribution according to the grade of malignancy is shown for each site in Table XIV.

TABLE XIV.—*Incidence of the Three Grades of Tumour According to the Part of the Breast Involved.*

Grade.	Cases per cent.						
				Quadrant.			
	Axillary tail.	Central.	Diffuse.	Upper outer.	Upper inner.	Lower outer.	Lower inner.
I	0	37	11	31	30	30	32
II	29	40	28	44	41	40	36
III	71	23	61	25	29	30	32
Total	100	100	100	100	100	100	100

It is evident that the poor prognosis for the small number of patients with axillary tail growths could be entirely accounted for by the preponderance of highly malignant tumours in this group, there being 71 per cent Grade III but no Grade I cases.

A study of those patients classified as having " diffuse " cancers shows a similar state of affairs. In this instance 61 per cent of the patients had Grade III tumours, whilst only 11 per cent were Grade I. The widespread nature of these growths and the poor prognosis would, therefore, appear to depend upon the high incidence of extremely malignant tumours.

Further examination of Table XIV reveals that, in contrast to the " axillary " and " diffuse " tumours, the proportion of the three types of cancer in the central and various quadrant positions of the breast is very similar. Hence, in these cases we cannot evoke the distribution of the tumours to account for the slight differences in the five-year survival rates.

With regard to the cases separated out according to inner and outer hemisphere involvement (Table XIII), the scatter of the cases in each group is again seen to be similar (Table XV).

TABLE XV.—*Incidence of the Three Grades of Tumour According to Inner or Outer Hemisphere Involvement of the Breast.*

Grade.	Cases per cent.	
	Inner Hemisphere.	Outer Hemisphere.
I	31	31
II	41	39
III	28	30
Total	100	100

Let us now reconsider the problem of site and prognosis, but this time taking into account the grade of malignancy. To avoid breaking the cases up into too many small groups, only inner and outer hemisphere growths will be studied. The results of this investigation are laid out in Table XVI. It is interesting to

TABLE XVI.—*Site, Grade and Prognosis.*

Grade.	Outer hemisphere.			Inner hemisphere.		
	Cases.	5-year survivors.		Cases.	5-year survivors.	
		No.	Per cent.		No.	Per cent.
I	76	65	86	29	21	72
II	100	47	47	37	15	41
III	67	18	27	28	8	29

note that site may be of some importance for Grade I carcinomas, the outlook being slightly worse when the inner half of the breast is involved. The difference for the intermediate cases is very small and of doubtful significance. Tumours of high grade malignancy are seen to have an equally bad prognosis whether they are situated in the inner or outer regions.

We conclude from the present investigation that, generally speaking, site of tumour in breast cancer exerts no striking effect on prognosis, apart from those patients in whom the growth involves a large part of the gland, and this is largely determined by the histological type of neoplasm (i.e. Grade III). On the other hand, the outlook for the women with centrally placed growths may be slightly less favourable than for those with quadrant tumours, presumably because of their close proximity to the sub-areola lymphatic plexus of Sappey. Cases of low grade malignancy may be influenced to a minor degree by the site of the growth, depending upon whether the sternal or axillary half of the breast is affected (Table XVI). The reason for this may be as follows. Site is of no consequence for Grade III tumours owing to their tendency to metastasize early—no matter where they are situated, rapid, wide-spread secondary deposits appear to be the rule. In contrast to this, Grade I tumours disseminate much less readily. Hence site becomes important, the nearer these growths are to inac-

cessible channels of spread (e.g. internal mammary chain), the greater the likelihood of such secondary involvement having taken place before the primary is removed.

SIZE OF TUMOUR AND PROGNOSIS.

Can the size of the tumour prove of any value in assessing outcome in mammary carcinoma ? Geschickter (1945) points out that this feature is seldom discussed, although it is usually agreed that cancer occupying the entire gland is most frequently hopeless. In the present series there were 18 cases in which the growth was described as being "diffuse" or occupying the "whole breast," and of these only 17 per cent survived five years. Such cases are now rarely seen. What of the prognostic significance of smaller, less advanced growths ? Here again, as with so many other aspects of breast cancer, opinions do not agree.

Kunath (1940) failed to find a correlation between size of tumour and prognosis. A similar result was obtained by Hoopes and McGraw (1942). On the other hand, Eggers, de Cholnoky and Jessup (1941) showed a five-year survival rate of 73 per cent for patients with neoplasms of 2 cm. or less. With an increase of size (3 to 6 cm.) the survivals dropped to 24 per cent, reaching 16 per cent for the largest growths (7 cm. or more).

In the present study information regarding the size of the tumour was available in 350 cases. These were separated out into three groups and the survival rates determined (Table XVII). An examination of this table reveals that the prognosis deteriorates as the tumour increases in size.

TABLE XVII.—*Size and Prognosis.*

Size.	Cases.	5-year survivals.	
		No.	Per cent.
1″ or less.	172	101	59
1″ – 2″	141	64	45
> 2″	37	12	32

So far, we have considered the cases as a whole, no allowance having been made for the different types of growth. The importance of taking this into account is obvious. For example, it would be fallacious to compare a small, highly malignant growth with one of large dimensions, but of low grade malignancy. Geschickter (1945) also refers to this point—"the size of the tumour is a reliable index of prognosis only if the pathological type is taken into consideration." It therefore follows that the better outlook for smaller tumours may result from a greater proportion of cases of low grade malignancy existing in this group. The converse would apply to the larger growths, here there being a preponderance of highly malignant examples. This, indeed, was found to be the case in the present investigation (Table XVIII). Of the tumours with a diameter of 1 inch or less, 37 per cent are classified as Grade I and 23 per cent as Grade III. On the other hand, in the case of growths of more than 2 inches diameter only 8 per cent belong to Grade I whilst 54 per cent are Grade III. When the diameter lies between 1 and 2 inches the incidence of these tumours is practically the same.

The distribution of cases as shown in Table XVIII once again emphasizes the importance of the histological type of growth in cancer of the breast. A further study must therefore be made in which attention is given to size and prognosis

TABLE XVIII.—*Distribution of Cases According to Size and Grade.*

Size.	Grade.	Cases.	
		No.	Per cent.
1″ or less	I	64	37
	II	68	40
	III	40	23
	Total	172	100
1″ – 2″	I	39	28
	II	67	47
	III	35	25
	Total	141	100
> 2″	I	3	8
	II	14	38
	III	20	54
	Total	37	100

according to grade of malignancy. Owing to the small number of patients with tumours over 2 inches in diameter, only two groups of cases will be considered instead of the original three—those with growths below and above 1 inch diameter. Table XIX reveals the five-year survivals according to size and grade. It is

TABLE XIX.—*Grade, Size and Prognosis.*

Grade.	Size.	Cases.	5-year survivals.	
			No.	Per cent.
I	1″ or less	64	49	77
	>1″	42	33	79
II	1″ or less	68	40	59
	>1″	81	27	33
III	1″ or less	40	12	30
	>1″	55	16	29
	Total	350	177	50

evident that size appears to be of no prognostic significance with regard to carcinomas of either low or high malignancy. Whether the tumours are small or large, the outlook is uniformly good in the former and bad in the latter group. On the other hand, an intermediate result is obtained for the intermediate cases (Grade II), the survival rate being practically halved in the presence of the larger neoplasms. In other words, the metastasizing power for Grade I and also Grade III cancers is independent of size. For growths classified as Grade II this power bears a direct relationship to the diameter, the larger the tumour the greater the likelihood of spread having taken place.

We consider that the conflicting opinions expressed regarding the relationship between size of tumour and prognosis in mammary carcinoma result from the study of histologically incomparable groups of cases.

PREGNANCY OR LACTATION AND PROGNOSIS.

It is now generally believed that breast cancer, when associated with pregnancy or lactation, is particularly virulant and bears a bad prognosis. This complication was not reported as being present in any of the cases in the present investigation. For the sake of completeness, however, brief attention will be given to this feature by referring to the work of other authors.

The gloomy outlook for these patients is well supported by Lee (1931) who, in a small group of 25 cases, finds only 8 per cent alive and well three years after operation. A larger investigation was carried out by Harrington (1937). He studied 92 examples of mammary carcinoma in pregnancy or lactation treated at the Mayo Clinic between the years 1910 and 1933. The five-year survival rate was 15 per cent compared with 44 per cent in the larger general series from the same centre. Axillary metastases occurred in 85 per cent of the patients (64 per cent in the general series), thus lending further weight to the view that the tumours of such cases are more malignant and spread more rapidly than do those which are not associated with pregnancy. The prognosis was practically hopeless for patients with involvement of the axilla, a mere 6 per cent surviving five years (28 per cent in the general series). On the other hand, pregnancy or lactation in the absence of this complication did not appear to effect the outcome adversely, there being 62 per cent five-year survivals compared with 72 per cent in the general survey.

More recently, Richards (1948) reports a five-year survival rate of 25 per cent for a small group of cases compared with 43 per cent in his general series. It appears that the very grave prognosis for these patients has been improved by the addition of irradiation to surgical treatment (Cade, 1950).

What is required now is a histological study to determine the incidence of the different types of breast cancer occurring in pregnancy. If the tendency is towards a high degree of malignancy, and this would appear to be the case from the clinical data, then a preponderance of Grade III cases is to be expected. Unfortunately, this view cannot be confirmed at the present time owing to the lack of material. It is intended, however, to undertake such a study at a later date. Meanwhile, we may refer again to Harrington (1937). This author graded the tumours of 80 of his cases according to the method of Broders (which is based on four grades), and found that practically all were of a high degree of malignancy ; in point of fact, there was not a single instance of a Grade 1 case and only 6 per cent belonged to Grade 2, whereas 25 per cent were of Grade 3 and 69 per cent of Grade 4. (Table XX.)

TABLE XX.—*Distribution of Cases in Pregnancy According to Grade of Malignancy (Harrington, 1937).*

Broders' Grade.	Cases per cent.
1	0
2	6
3	25
4	69
Total	100

It is of interest to note that pregnancy following the adequate control of a previous mammary carcinoma does not appear to influence the outlook adversely. Thus in the investigation by Harrington (1937) there were 55 cases in such circumstances, and 79 per cent were alive at the end of five years. The percentage of survivals for patients with and without axillary metastases was 57 and 97 respectively. But in spite of these excellent results, Harrington considers it inadvisable for women who have been treated for breast cancer to undergo a subsequent pregnancy.

CONCLUSIONS.

A number of clinical factors which have been claimed to influence prognosis in carcinoma of the breast have been examined. We believe that much of the controversy which centres around these problems has developed as the result of studying groups of cases which are not comparable from the histological point of view.

The importance of the microscopic appearance of the tumour in classifying and assessing the prognosis of patients with breast cancer has been stressed elsewhere (Bloom, 1950). This feature has been taken into account in the present investigation, and from the results we conclude that, in spite of widely held views to the contrary, the age of the patient, the duration of symptoms and the site of the primary growth exert little or no influence on the ultimate outcome. A prognostic factor of far greater importance would appear to be the type of tumour as determined by a histological grading system—a factor which, unfortunately, is almost entirely neglected at the present time.

From this and our previous work the assistance that can be given by the pathologist to those dealing with the clinical aspects of breast cancer is obvious. We consider that many of the problems which have arisen concerning the results of treatment of this disease depend upon the inaccurate grouping of cases. It is hoped that the wider use of morbid histology will help to reduce this source of error to a minimum, and so enable us to assess the true merits of the various lines of therapy ; this is our ultimate aim.

SUMMARY.

1. The present work is a continuation of the investigation into the problems of prognosis in mammary carcinoma reported recently in this Journal (Bloom, 1950).

2. The relationship between the age of the patient and prognosis was studied. No significant differences in outcome were revealed in the various age groups, the younger women faring no worse than the older ones. These findings were confirmed by the fact that the incidence of tumours of low and also of high malignancy in the various decades was practically the same.

3. It is useless to try to assess the effect of the delay in seeking treatment without reference to the histological type of growth involved. For example, delay is of little importance for patients with highly malignant (Grade III) tumours ; it does not seem to matter whether such cases attend for treatment early (less than 6 weeks) or late (6 to 12 months), the prognosis is equally bad. On the other hand, the time factor appears to influence the outlook of women with growths whose behaviour is essentially more benign (Grade I). In this type of case a loss of more than 6 months means a substantial fall in the survival rate.

4. The site of the tumour in the breast was, generally speaking, found to exert no striking effect on prognosis. However, it is possible that the outlook for patients with growths of low grade malignancy may be influenced slightly by this feature, depending on whether the medial or lateral hemispheres are involved.

5. When the patients were considered as a whole the size of the primary growth was found to influence prognosis, the larger the tumour the lower the survival rate. It is in the intermediate grade of tumours that these differences are especially found, and it is cases of this grade that are largely responsible for the general result. Size was evidently of no prognostic importance for women with tumours of a low and also a high grade of malignancy. It did not appear to matter whether the growths were small or large, the outlook was uniformly good in the former and bad in the latter group. In contrast to this, the prognosis for neoplasms of an intermediate degree of malignancy showed marked deterioration with increase of size.

6. Brief reference has been made to the progress of cases of breast cancer when associated with pregnancy or lactation.

Note by T.E. Cowan, Esq., F.C.I.S., F.R.S.S.: The inferences drawn from the tables shown are statistically sound.

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THE PRINCIPLES OF TREATMENT BY RADIOTHERAPY IN BREAST CARCINOMA.

R. McWHIRTER.

From the Department of Radiotherapy, Royal Infirmary, Edinburgh.

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In only a minority of cases is the disease still confined to the breast by the time the patient is referred to a large general hospital. The surgical results are good so long as the disease is confined to the breast, but immediately the disease extends beyond the breast the results become much poorer. Axillary spread is, of course, common and, in the past, surgical efforts have been concentrated on this route of spread. That this is not the only route of spread, however, has been clearly demonstrated by Handley and Thackray (1947), who have shown that in a high proportion of cases where there is axillary involvement there is also involvement of the glands along the internal mammary artery. Treatment of this route of spread by surgery is not at present possible. In addition the supraclavicular glands are involved in 33 per cent of the cases where the axillary glands are involved and, while surgical removal of the supraclavicular glands may be attempted, the value of this procedure is extremely doubtful. For the majority of cases of breast carcinoma it therefore follows that surgery is unlikely to be successful unless supplemented by radiotherapy.

Success in radiotherapy is dependent on the sensitivity of the tumour to radiation. Unfortunately breast carcinoma is only moderately sensitive, and improved results can only be expected if the radiotherapy is carefully planned.

It is not the purpose of this paper to give a detailed account of the treatment of a patient by radiotherapy, but rather to emphasize the main principles in the treatment of breast carcinoma by radiotherapy. There are five main principles :

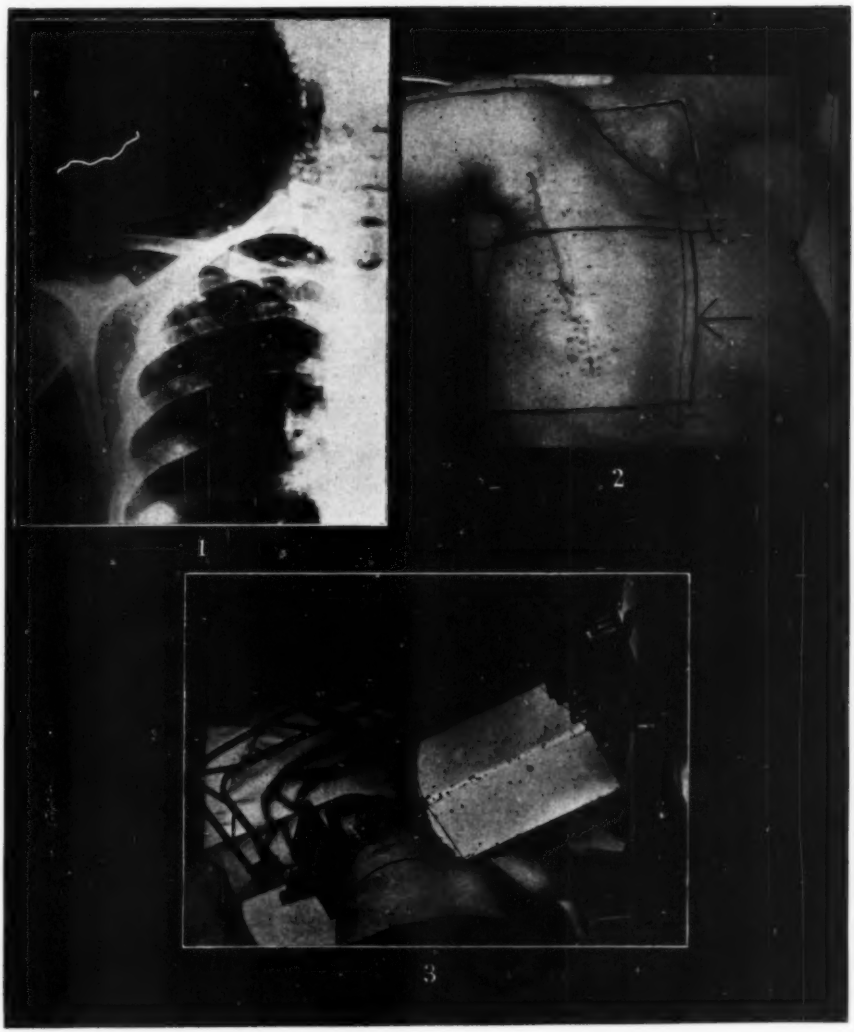
1. *The axillary and supraclavicular glands must be treated as one continuous chain.*

In the past it has been customary to regard the supraclavicular glands as being somewhat separate and distinct from the axillary glands. I believe the supraclavicular glands are best regarded as representing the proximal group of glands which accompany the axillary vessels.

If a finger is placed in the apex of the axilla at the time of a radical operation, it can be easily demonstrated that the apex in fact lies almost deep to the supraclavicular region.

In tuberculosis the disease may readily spread from the neck glands to the axillary glands, as is demonstrated in Fig. 1. This radiograph shows the two main groups of glands in the neck—the carotid chain accompanying the carotid vessels and the posterior cervical chain which lies along the anterior border of the trapezius. It will be noted that both chains are continuous with the glands of the axilla, and that the medial group of supraclavicular glands form the proximal group of the axillary chain.

Further proof of the continuity of the neck and axillary glands is shown in



carcinoma of the thyroid, for if the glands in the neck are involved it is common to find that there is also axillary involvement.

Continuity with the posterior cervical chain is readily demonstrated in patients suffering from carcinoma of the nasopharynx where the posterior cervical chain is commonly involved. If the lower glands of the posterior cervical chain are involved there is nearly always involvement of the axilla.

In the above examples spread downwards has occurred, but it may be assumed that if downward spread can take place the reverse is also possible. As has already been mentioned when the glands in the axilla are involved in breast carcinoma the supraclavicular glands are involved in 33 per cent of cases, and there can be little doubt that in a proportion of cases the supraclavicular glands have become involved by upward spread from the axilla.

It follows therefore that the axillary and supraclavicular glands must be treated as one continuous chain of glands, and the only satisfactory method of doing so is by the use of two directly opposed fields—one field placed in front and the other placed posteriorly as in Fig. 2.

Irradiation of the axilla by a field applied to the base of the axilla and irradiation of the supraclavicular glands by a separate field is unlikely to meet with success because the intervening glands are not treated. It has been stated by some workers that it is difficult to irradiate the supraclavicular region successfully. I believe that in many cases the reappearance of glands in the supraclavicular region has not been due to any difficulty in treating this area, but is due to reinfection of the supraclavicular glands from involved glands high up in the axilla which received no radiation at all.

2. *The internal mammary glands must be treated in continuity with the chest wall.*

This is best done by glancing fields so as to avoid lung damage. The medial glancing field should be placed beyond the midline so as to include within the irradiated area the glands along the internal mammary artery on the side affected (Fig. 3). By using large fields the dose distribution over the area can be made fairly uniform. The use of bolus further assists in obtaining a homogeneous distribution.

The usual separation between the glancing fields is 16 cm., and in radical treatment it is rarely necessary to exceed this distance. If the skin involvement is more extensive and all the diseased area cannot be satisfactorily included within those limits, only palliative treatment is indicated.

It will have been noted that the total area requiring irradiation has been divided into two sections. Every effort is made to avoid a gap between the two sections, and experience has shown that it is uncommon for recurrence to take place along the line of junction. Ideally, of course, it would be better to irradiate the whole of the area to be treated without any division. Unfortunately this cannot be achieved because the axillary and supraclavicular glands cannot be satisfactorily treated except by means of directly opposed fields. (Carcinomata of the axillary tail of the breast do, however, call for some modification of the usual technique.)

3. *Hard quality radiation is essential.*

With radiation of long wave-length much more energy is absorbed by bone than by soft tissue. Not only may this result in bone necrosis, but any tumour

cells situated in the path of the beam distal to the bone will receive less dosage than might be expected from isodose curves obtained by measurements taken in a uniform medium.

In Edinburgh we use a beam generated at 250 kV. constant potential and filtered by a " triple " Thoraeus filter (1·0 mm. steel (tube window), 1·5 mm. tin, 1·0 mm. aluminium), giving a half value layer of 3·7 mm. copper. Further proof of the value of this quality of radiation was demonstrated during a breakdown of the main apparatus, when it became necessary to treat patients on a 220 kV. pulsating tension apparatus with only 1 mm. copper filtration. The half value layer was 1·6 mm. copper. Axillary glands, palpable before treatment was commenced, did disappear, but as a general rule the disappearance was only temporary, and recurrence took place in a high proportion of these cases at about six months after treatment had been given.

4. *Adequate dosage must be delivered throughout the whole of the treated area.*

We aim at a minimum tumour dose of 3750 r in three weeks. The maximum dose varies according to the thickness of the patient, but is not allowed to exceed a maximum of 4500 r. On this account if a patient is stout and the minimum tumour dose cannot readily be achieved it is better to carry out a radical operation.

5. *Only one course of treatment should be given.*

Repeated courses of treatment at intervals of three to six months are quite illogical if the intention is to give radical treatment. Repeated courses imply that malignant cells are deliberately left behind for further treatment at a later date.

TABLE I.—*Total Cases referred* 1941–45 = 1345.

Operable 60 per cent.		Inoperable (radical operation) 40 per cent.	
Axillary glands negative	Axillary glands positive	Advanced locally	Distant metastases
24 per cent	36 per cent	25 per cent	15 per cent
Axillary dissection unnecessary	Disputed group	Radical mastectomy contra-indicated	Radical mastectomy contra-indicated
Simple mastectomy + X-rays	Simple mastectomy + X-rays	Simple mastectomy + X-rays or X-rays alone	No treatment or palliative treatment
5 year Survival rate 89 per cent	5 year Survival rate 44 per cent	—	—
All operable cases (757) 5 year survival rate 62 per cent		All locally advanced cases (389) 5 year survival rate 29 per cent	—
All cases without evidence of distant metastases (1146) 5 year survival rate 50·5 per cent			
Every case (treated and untreated) coming to the hospital (1345) 5 year survival rate 43·7 per cent			

ASSESSMENT OF THE VALUE OF THE TECHNIQUE DESCRIBED.

In Edinburgh the policy adopted has been to carry out only a simple mastectomy and to treat the axilla entirely by radiotherapy. The technique described above has therefore been amply tested. Table I shows the results obtained.

The frequency of axillary involvement in the operable cases (Stages I and II Manchester Classification) has been estimated from an earlier period when radical mastectomy was practised, and is in general agreement with other published figures.

It will be noted that results have been presented for cases with histologically negative and histologically positive glands, where only a simple mastectomy has been performed. Those figures have been obtained where it has been possible to remove, at the time of the simple mastectomy, a gland from the subpectoral region or from low down on the medial wall of the axilla.

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STUDIES IN ANTIBODY RESPONSE OF MICE TO TUMOUR INOCULATION.

P. A. GORER.

*From the Department of Pathology, Guy's Hospital Medical School, London, S.E.*1.

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It has been shown that following the inoculation of neoplastic tissues or of blood from one pure strain of mice into another, a variety of different types of antibody might be formed (Gorer, 1942, 1947). Confining our attention to the reactions obtained with the erythrocytes of the donor strain, it was found that three different types of antibody could be demonstrated: the ordinary agglutinin, an agglutinin that was active in high concentrations of normal mouse serum, and "blocking" antibodies. The last could only be demonstrated by their inhibiting action with the ordinary agglutinins. The albumin technique of Diamond and Denton (1945) was not applicable, since mouse cells are lysed by albumin. Attempts to produce a Coombs serum (Coombs, Mourant and Race, 1945) by inoculating rabbits with normal mouse plasma were not successful. This is not without interest, since it may indicate that the blocking antibody globulin of mice is absent from normal serum, or present in too low a concentration to elicit antibodies in the rabbit. Such a state of affairs is not without precedence. Thus van den Ende (1940) showed that guinea-pigs inoculated with normal rabbit serum would not give precipitation with purified anti-pneumococcal antibody. Similarly physico-chemical studies in horse sera show the presence of a new type of molecule following immunization (Green, McKalk, Kapnick and Fahey, 1939; van der Scheer, Wyckoff and Clarke, 1941). Whilst these questions will have to be cleared up, it was decided to attempt other methods first.

It was thought that some animal sera might enhance agglutination, and a few experiments were performed whilst the author was at the Roscoe Jackson Laboratory with rabbit and horse sera. In both these cases it was obvious that the absorption of natural anti-mouse agglutinins from the undiluted sera would be a formidable task. The work of Waaler (1939), Wiener (1944, 1945) and Boorman and Dodd (1947) suggested the value of human serum in enhancing agglutination, and the results obtained thereby are reported below.

Materials and methods.

The mice used were of well-known strains, Strong A, C57 black, Bagg Albino (Strain C), C3H and CBA. Methods of bleeding, etc., have been given in the papers quoted (Gorer, 1938, 1947). A sarcoma arising in the stroma of an A strain mammary cancer known as S/Ay was obtained through the kindness of Dr. Ludford (Ludford and Barlow, 1945), and maintained by the ordinary trocar

and cannula technique. A lymphogenous leukosis in strain C57 blacks was obtained by the author following treatment with 9:10-dimethy-1:2-benzanthracene. When first obtained in 1945 it was maintained by the intraperitoneal inoculation of saline emulsions of infiltrated organs. Since 1946 it was kindly maintained by Professor Haddow and Dr. Carr at the Chester Beatty Research Institute, and was transmitted subcutaneously by trocar and cannula technique. Since obtaining it again it has been transmitted by means of cellular emulsions, sometimes inoculated subcutaneously, sometimes intraperitoneally. When the latter route is used it can be seen that the gross morbid anatomy has radically changed. Previously the picture was typical of lymphatic leukaemia, with generalized glandular enlargement, enlarged spleen, etc. Now the behaviour is more that of a lymphosarcoma, with diffuse infiltration of the peritoneal tissues and a terminal ascites.

If the A strain tumour is inoculated into blacks, a tumour appears that regresses in about 3 weeks. In Bagg albinos it follows a similar course in about 85 per cent of cases; in the remainder persistent growths occur. Such mice were usually bled when they began to appear ill; however, a few were left and died 2 months or more after inoculation, living more than twice as long as A strain mice.

The leukosis E.L.4 has been inoculated subcutaneously into mice of strains A, C3H and CBA. The behaviour in all is much the same. A tumour is visible in 7 days, which has disappeared by the 14th day.

Except for the Bagg albinos with persistent growths, mice were bled 7 to 10 days after 3 or more inoculations given at about 10-day intervals. As a general rule pooled sera from 4 to 6 mice were used in experiments. The technique of bleeding has been described previously (Gorer, 1938, 1947).

Human serum was selected simply on the grounds of availability. It so happened that a considerable number of AB sera had been obtained for Rhesus grouping, so that this type has been used fairly frequently. Group B serum has not been used since that in store was needed for other purposes, and there was no easily available donor. The serum was absorbed undiluted in the cold room or refrigerator (from 2° to 4° C.). The cells of any mice of any strain may be used. These were washed twice. For the first absorption about 2 volumes of serum was used for each volume of packed cells, for the second absorption the relative volume of cells was increased, being in the ratio of about 3 volumes of serum to 2 of cells, each absorption being for about an hour. As will be reported below, the results differ when one uses heated or unheated serum. In the latter case it is very difficult to avoid haemolysis on absorption.

All sera were stored at—20° C. Human sera stored in this way prior to absorption are highly active after several months. It is not known how long they will keep following absorption, since they were used very rapidly. The vagaries of mouse iso-antibodies on storage have been referred to in papers mentioned above (Gorer, 1938, 1947). Those studied here are better, some being good after a month.

The technique of performing the agglutination tests was generally similar to that described in previous publications (Gorer, 1938, 1947). However, latterly the practice of spinning slowly prior to reading has not been used. Slightly higher titres may be obtained if this is done, but trouble may be experienced in obtaining an end-point due to slight traces of antibody in the human serum.

If the tubes are not spun readings may be made after about 90 minutes at 37° C., whilst if the centrifuge is used this may be done earlier.

RESULTS.

(a) *With heated human serum.*

When the sera from C57 blacks hyper-immunized with the A strain tumour were tested against the corresponding cells, ordinary saline agglutinins were found, as was to be expected from previous experience; however, it was found that when human serum was used as a vehicle both for serum dilutions and suspending the red cells there was a very obvious enhancement of agglutination—a result in entire agreement with that obtained by Boorman and Dodd (1947). Encouraged by this finding it was decided to investigate the sera of Bagg albinos immunized with the same antigen. When tested by the previous techniques only one individual gave any agglutination; the solitary exception surprisingly gave a high titre of ordinary saline agglutinins.

TABLE I.—*The Effect of Heated Human Serum and Mouse Serum on Anti-A Strain Bodies from Bagg Albino Mice.*

Antibody from 2 mice with persistent graft of A strain sarcoma of over 8 weeks' duration.
Human serum P.A.G. Mouse serum C3H. Tested on A cells.
Spun 1 minute after 2 hours 20 minutes at 37° C.

Cells and antibody in—	Control.	Agglutination with dilution of antibody 1 in—											
		2	4	8	16	32	64	128	256	512	1024	2048	4096
Saline . .	— .	—	—	—	—	—	—	—	—	—	—	—	—
Mouse serum .	— .	—	—	—	—	—	—	—	—	—	—	—	—
Human serum 1/1	tr. .	±	++	++	ac	c	c	c	c	c	ac	++	+
,, ,, 1/2	— .	—	—	—	—	+++	ac	c	ac	+++	+++	±	±
,, ,, 1/4	— .	—	—	—	—	tr.	++	+++	++	++	+	tr.	?
,, ,, 1/8	— .	—	—	—	—	?	±	+	±	tr.	?	—	—

c and ac = Complete and almost complete agglutination respectively.
tr. = trace.
± to +++ = Ascending strength of agglutination.

In Table I is shown the result obtained with the pooled serum of two mice with large persistent growths of over two months' duration. It will be noted that mouse serum fails to bring about agglutination, whereas human serum does so to a fair dilution, albeit with a marked pro-zone.

It will be noted that the controls with undiluted serum were not entirely satisfactory. In several subsequent experiments these were done in quadruplicate. It was a common finding that one or two would show some clumps whilst the rest would be satisfactory. It is doubtful if this phenomenon is due to unabsorbed antibody. If absorption has been inadequate one expects to find numerous small clumps; here the clumps were few and large. A further objection to the use of serum as a vehicle for all operations is the frothing that is apt to occur when serial dilutions of antibody are being made. Since a final dilution of 50 per cent human serum seemed to give adequate results, this has been used in most subsequent work. A further point that is noticeable, when lower dilutions of human serum are used, is that there is an apparent optimum agglutination

with an antibody concentration of 1 in 128. This optimum has been found remarkably constant in Bagg albinos with persistent growths.

Once it was decided that human serum at a final concentration of 1/2 had certain advantages, experiments were performed to see if the order in which the ingredients were mixed had any effect on the result. It was found with various systems that a decidedly better result was obtained if the cells were suspended in serum prior to contact with the antibody. In most subsequent work the cells have been suspended in pure serum and the antibody dilutions performed in saline.

TABLE II.—*Variation in Potency of Human Sera.*

Anti-A agglutinins same source as Table I.
Anti-C57 black from 6 CBA's inoculated 5 times with leukosis E.L.4.
Anti-A system read after spinning.
Anti C57 system not spun.
Cells in serum ; antibody dilutions in saline.

A cells in serum from—	Control.	Agglutination with dilution of antibody 1 in—											
		2	4	8	16	32	64	128	256	512	1024	2048	4096
P.A.G. . .	—	—	—	—	tr.	+	ac	c	+++	++	++	+	±
"Ors" . .	—	—	—	—	tr.	±	+	+++	+	+	tr.	tr.	tr.
C57 B1, cells in serum from—													
Miss B— . .	—	+++	+++	ac	+++	+++	+	tr.	—	—	—	—	—
Mrs. E— . .	—	?	±	±	tr.	—	—	—	—	—	—	—	—

Boorman and Dodd (1947) found fairly striking variations in the enhancing power of various samples of human sera. Far fewer have been tested here. Table II shows two pairs of sera tested on different systems. The top pair shows the usual range of variation. Both of these sera are obviously usable. The lower pair are of interest from two points of view. The second serum, "Mrs. E—," is virtually useless, the only sample that has been found to be so.

Of greater interest is the fact that the antibody in the second case is reacting with the cells of C57 blacks. Previously the author has made several attempts to demonstrate such antibodies ; the highest titre that had been obtained was two from C3H mice titrated in normal mouse serum. That titre is not quite so good as that obtained from the same batch of mice when they were bled following the 4th injection ; similar findings have been made with other systems. The results obtained with C3H sera are shown in Table III. Only one sample of serum from immunized A's has been tested so far. The titre was 512 in 50 per cent serum (Miss B—) without spinning. It would appear that all three strains give much the same titre with E.L.4. Furthermore these are much the same as those obtained following hyper-immunization with A strain antigens.

(b) *A comparison of the results obtained with heated and unheated human sera.*

As was stated previously, when fresh unheated human serum is absorbed with mouse cells, considerable haemolysis usually results. No reliable method has yet been found of overcoming this ; it may prove necessary to perform the

first absorption with washed stromata or possibly some blood-free organs. Strangely enough the serum " Ors " shown in Table II did not cause haemolysis on absorption.

TABLE III.—*A Comparison of the Effects of Heated and Unheated Human Sera.*

Anti-A from Bagg albinos with large growths. Tested on A cells in heated and unheated serum. Antibody dilutions in saline.
Anti-C57 black from C3H inoculated 3 times with leukosis E.L.4. Antibody dilutions in saline.

	Control.	Agglutination or lysis with dilution of antibody 1 in—								
A cells in—		2	4	8	16	32	64	128	256	512
Unheated serum	—	T	T	T	H	H	h	h	—	—
Heated serum	—	—	—	—	?	++	+++	++	++	+
C57 black cells in—										
Unheated serum	—	H	H	H	H	H	H	h	h	?
Heated serum	—	±	+++	+++	ac	+++	+++	+++	+	—

T = Total lysis. h = Weak lysis.
H = Strong lysis. — = Negative reactions.
Symbols for agglutination as in Table I.

Table III again shows two systems, Bagg albino anti-A, and C3H anti-C57 black. In the former case the serum was obtained from mice with persistent growths, in the latter from mice bled on the 7th day following 3 injections of E.L.4.

It will be noticed that different symbols have been used for lysis and agglutination; the minus sign means that both reactions are negative. It will be seen that with the anti-A serum the haemolytic titre is considerably lower than the agglutination titre, in spite of the fact that the serum " Ors " is a rather poor enhancer of agglutination (Table II). However, there is no pro-zone with haemolysis—a result confirmed with five other sera. A further important point is that above the limits of haemolysis there is no agglutination with unheated serum.

The significance of pro-zones in agglutination has been discussed elsewhere (Gorer, 1947; Race and Sanger, 1950). It would seem that in this case we are dealing with a distinct type of antibody. Under the conditions of experiment shown in Table III we may say that it is haemolytic, but gives no agglutination.

The C3H antibody shows a very slight pro-zone for agglutination. Haemolysis in this case was never complete, although quite obvious. In this case the lytic and agglutination titres were about the same. An almost identical result was obtained with CBA anti-C57 black serum. Under unfavourable circumstances one may get no lysis with unheated serum, but simply inhibition of agglutination, whilst the same system employed with the human serum heated will give a positive result. It would appear that for general purposes heated serum is likely to be the more useful. However, unheated serum should not be neglected. It is quite possible that some sera will give a zone of agglutination that is very narrow or non-existent; if such do occur lysis might well give a good indication of the presence of an antibody.

In the light of the above results it was decided to test the effect of guinea-pig complement on cells sensitized with different types of antibody directed against strain A cells. In the case of the ordinary " saline " agglutinin (from C57 blacks) haemolysis occurred. In the case of the partial antibody from Bagg albinos

strong specific agglutination occurred in a certain proportion of cases. Unfortunately there was a very considerable variation between different batches of complement. If the haemolytic titre as tested with the ordinary sheep cell system was below 1/100 agglutination was never obtained. Unfortunately the converse was not true, and no rapid way has been found of finding whether the complement is going to be useful. In those cases where good agglutination did occur, the result was rather like that obtained with fresh human serum. There was no pro-zone, but the titre was only about 64 to 128 as against 4000 or more with heated human serum. The phenomenon is probably an example of the conglutinin reaction of Bordet (Hole and Coombs, 1947), and is being studied further. The antibody demonstrated is probably that which gives lysis, but no agglutination in human serum.

Before passing to a general discussion of the results, mention may be made of the possible existence of natural iso-antibodies detectable by means of human serum. It was shown previously (Gorer, 1947) that such bodies might occur in the sera of C57 blacks and Swiss mice. The titre was usually very low, and their existence could only be demonstrated in high concentrations of mouse serum. No systematic search has been made in this case, and no certain example has yet been found with the new technique. If they do occur it is unlikely that they can cause any confusion with immune antibodies such as those demonstrated here.

DISCUSSION.

It now seems possible to state that at least four types of antibody can be demonstrated as being directed against the red cells of mice. There is the " saline " agglutinin, the " mouse serum " agglutinin, and the " human serum " agglutinin. Finally we have to account for the pro-zone found in connection with the last of these. This is probably due to a fourth type of antibody that does not agglutinate in human serum, but does cause lysis. The picture as a whole is remarkably like that found in Rhesus iso-immunization in man. The various orders of antibody to be found here have recently been reviewed by Race and Sanger (1950). To complete the picture it will be necessary to devise a Coombs test for mice, since certain human iso-antibodies have only been recognized thereby.

A comparative study of the enhancing power of human serum on the murine and human Rh antibodies is in progress by the present author and Dr. D. A. Osborn. It seems that the same agent is operative in both, but that the murine systems are far more sensitive to its action.

Whilst much remains to be elucidated concerning the role of these antibodies in the destruction of grafts, there are certain ways in which an extension in our ability to detect iso-immune reactions is of value to oncology. The first of these concerns the ability of tumours to grow in foreign strains. It was shown (Gorer, 1947) that tumours persisting for some time in a strange host did, in fact, induce high titres of antibodies, much the same as those shown here, but these growths were not carried through successive transfers in the strange host. In the earlier stages of this work it was of importance to show that the tumour cells contained the same antigen as the erythrocytes (which was, of course, shown by absorption), and that the antibodies were induced by the tumour cells, and not by included erythrocytes. To those acquainted with the types of antibody responses given by the various tissues, this last hypothesis would now seem farcical (Kaliss and

Jay, 1950). Nevertheless as additional controls, attempts were made to demonstrate antibodies after two transfers of an A strain sarcoma (known as S15) in strange hosts (Gorer, 1938). With sufficient care this may be done, but the difficulties of doing so with the growth used then are of more interest. If it was removed from a mouse of strains CBA or C57 black at a time before regression commenced and put back in the A strain, it would grow, but only after a very prolonged latent period. If it was transferred to another individual of one of the other two strains, the result was much the same as that occurring in an immunized animal, namely a very rapid destruction of the graft. It seems that these results can be explained by assuming that the sarcoma cells were heavily sensitized with antibody before they showed any obvious damage. The results of transfer back to the A strain are generally similar to those obtained (Gorer, 1942) when leukaemic cells were suspended in immune sera prior to inoculation in the same strain. In the resistant strains the immediate destruction of the grafts is presumably due to antibody carried over in the one case and present in. or rapidly mobilized by, the hosts in the second. The behaviour of Ludford's tumour SA/y in the Bagg albino strain may be shown to be different, at least under certain circumstances. It was carried through three transfers in this strain, the antibody titres being the same at the first and last of these (over 4000). Clearly there was no loss of antigenicity, and it seems justifiable to deduce that high resistance to the action of antibodies is of prime importance in the ability to grow in foreign strains.

Another field in which improved serological techniques may be of value is in the assessment of results following inoculation of allegedly cell-free material. The simplest possible case is when material from strain X is found to give rise to a tumour in strain Y (where X and Y are any two strains). If this growth is found to be specific for the latter strain it does not matter very much if the initial material was cell-free or not. The case would be strengthened by a serological demonstration that this was indeed " Y " tissue, but it could not be regarded as essential. However, this happy state of affairs might not occur for two reasons. In the first place the tumour may be one that grows in many strains. Secondly, it is now common knowledge that the milk agent shows a marked predilection for certain strains (Bittner, 1945 ; Heston, 1945), and conditions might well arise in which success was only obtained with certain hybrids derived from the donor strain in which ordinary grafts would take. There are various ways round both these difficulties, and the result obtained following successive transfers of Ludford's tumour to Bagg albinos is a case in point. The original donor was a Bagg albino mouse that had been treated with 9:10-dimethyl-1:2-benzanthracene some weeks before inoculation with the A strain tumour. When killed the primary growth was tiny, but there was a mass over a centimetre in the axilla. Clearly this might have been either a metastatic A strain tumour or a Bagg albino tumour. There were very few A's available at the time, and it was decided to confine experiments to the Bagg strain. It took in about 50 per cent. This was suggestive, but not conclusive, since undoubted spontaneous tumours may sometimes do the same, especially in early transfers. However, the nature of the antibody response at the third transfer left no doubt that this was still A strain tissue. The serological study of tumours arising in heterozygous mice is a problem of importance from more than one point of view, but it can scarcely fail to be of some value to that just considered.

SUMMARY.

The employment of suitably absorbed human serum as a vehicle enables one to detect antibodies where previous methods have failed.

If heated serum is used one observes haemagglutination, with or without a considerable pro-zone.

With fresh unheated sera one obtains haemolysis, but no agglutination. The lytic action did not show a pro-zone, but with weak antibodies one may get no detectable agglutination or haemolysis.

There is some variation in the enhancing power of human sera, one being found that was useless.

One obtains a more satisfactory result if the cells come in contact with human serum prior to contact with antibody, the most convenient system for general use being obtained with the cells suspended in human serum and antibody dilutions carried out in saline.

Fresh guinea-pig serum may enhance agglutination, but the results are irregular.

There appear to be at least four types of antibody formed in response to tumour inoculation in mice: ordinary saline agglutinins, agglutinins active in high concentration of mouse serum, agglutinins inactive in mouse serum, but active in human serum, and antibodies giving a pro-zone in heated human serum, but giving lysis with unheated human serum. Those enhanced by guinea-pig complement may be identical with the last of these.

Further evidence has been obtained in support of the hypothesis that the ability of a tumour to grow in a foreign strain is due to a high resistance to the action of antibodies.

The value of serological technique to other oncological problems is discussed in the text.

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THE EFFECT OF FREEZING AND FREEZE-DRYING ON THE TRANSPLANTATION OF SARCOMA 37.

P. T. J. C. P. WARNER AND J. V. T. GOSTLING

From the Bland-Sutton Institute of Pathology, The Middlesex Hospital, London, W.1.

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RECENTLY, Gye and his colleagues (Gye, 1949 ; Gye, Begg, Mann and Craigie, 1949 ; Mann, 1949*b*, 1949*c* ; Mann and Dunn, 1949) removed tumours from mice, exposed them to a temperature of — 79° C., and stored them at that temperature. They also dried them at room temperature and from the frozen state. Samples of tumour tissue after treatment in any one of these ways were inoculated into mice and tumours grew from a proportion of the inoculations. At the same time Mann (1949*a*) showed that mouse embryo tissue, which normally grew on inoculation into mice, would not do so after freezing at — 79° C. From this it was assumed that all cells, neoplastic or normal, were destroyed by the freezing treatments applied to them. As a result of this it was postulated that a virus-like agent was responsible for the tumour-producing activity of neoplastic tissue subjected to low temperature.

Somewhat similar claims were made as early as 1908 (Salvin-Moore and Walker, 1908 ; Salvin-Moore and Barratt, 1908 ; Gaylord, 1908), but the tumour tissue was subjected to low temperatures only ; drying was not used. However, Sugiura and Benedict (1927) noted that it was possible to transmit mammalian tumours with dried tissue. Subsequently, several reports appeared stating that tumour tissue exposed to low temperatures retained its tumour-producing activity (Cramer, 1930 ; Breedis, Barnes and Furth, 1937 ; Barnes and Furth, 1937 ; Breedis and Furth, 1938 ; Mider and Morton, 1939 ; Klinke, 1940 ; Breedis, 1942 ; Passey and Dmochowski, 1950). Some of these workers produced evidence that, among both normal and neoplastic cells, some types did and some did not survive exposure to low temperatures.

The contentions of Gye and his colleagues (Gye, 1949 ; Gye, Begg, Mann and Craigie, 1949 ; Mann, 1949*a*, 1949*b* ; Mann and Dunn, 1949) have been subjected to criticism on the grounds that they were based on insufficient evidence. One of the most recent and competent reviews of the subject is by Hirschberg and Rusch (1950). They conclude that the results do not prove that a virus was present in the tumours studied, but that they do not necessarily exclude it.

Passey and Dmochowski (1950), however, using various mouse tumours, including Sarcoma 37, were unable to demonstrate virus, by one of the usual methods, in tumour tissue which had been exposed to low temperatures. They also review previous work.

In view of the conflicting conclusions arrived at in this field, it was considered important to investigate the subject further. This paper deals with the application of quantitative methods, as suggested by Craigie (1949*a*), to Sarcoma 37 both before and after freeze-drying and exposure to temperatures below — 75° C.

MATERIALS AND METHODS.

Sarcoma 37.—This was obtained from the Courtauld Institute of Biochemistry, where it had been maintained in serial passage in albino mice.

Animals.—Albino mice were obtained from a reputable dealer. They were used at the age of 4 to 8 weeks and were weighed in groups before use. The average weight of the mice used for each experiment ranged from 16 to 26 g. Each mouse was earmarked.

Preparation of sarcoma mince.

Mice, bearing on their backs subcutaneous tumours, were killed, dipped in lysol and pinned, face-down, on a board. The size of the tumours ranged from 3 × 2 cm. to 0·5 × 0·8 cm., most being about 2 × 1 cm. The skin was cut at some distance from the tumour and around approximately three-quarters of its perimeter. The edges of the cut skin were everted and seized at two or three places by artery forceps. An assistant held the forceps and pulled the skin upwards and outwards to reveal the tumour. Sterile instruments were taken, and the tumour or the greater part of it was dissected away from its surroundings and placed in a weighed sterile Petri dish. A number of tumours were collected in this way. The Petri dish, containing the tumours, was then weighed. About 10 g. of tumour tissue was found to be a convenient amount. This was usually obtained from between 5 to 10 mice which had been inoculated with one minim (0·06 ml.) of minced tumour tissue 10 to 14 days previously.

After weighing, the tumours were cut up as finely as possible with scissors. The material was then minced in a pressure mincer similar to that described by Craigie (1949*b*), plungers with grooves of depths 0·02 in. and 0·005 in. being used. The fine mince was then placed in a 10 ml. syringe, and some of it was cultured on a blood agar plate.

Titration of tumour-producing activity of sarcoma mince.

The tumour mince described above was diluted in tenfold serial steps in physiological saline. The undiluted material and the dilutions were then inoculated in doses of 1 minim (0·06 ml.) at 4 different sites on each mouse used. A dose of 1 minim was chosen, for the sake of convenience, as the 1 ml. syringes used for inoculation were graduated in minims. The number of mice used for each dilution varied from experiment to experiment. The usual number was either 3 or 5, more frequently the latter.

The mice were examined at least twice a week and the number of progressively-growing tumours were recorded. From the results obtained the dose of tumour mince which would have produced tumours in 50 per cent of mice was calculated by the method of Reed and Muench (1938). This is referred to as the TPD50 (TPD = tumour-producing dose) of the mince. For the sake of clarity and convenience the negative logarithm of the TPD50 is shown in the results and is called the " TPD50 index."

Quick freezing of sarcoma mince.

Quantities of 1 ml. of sarcoma mince were fed, by means of a syringe, fitted with a long needle, into ampoules (6 mm. bore stems, 15·5 mm. diameter bulbs, obtainable from W. E. Edwards & Co., London). Each ampoule in turn was

attached by a short stout piece of rubber tubing to the spindle of a 1/10 h.p. electric motor (Fig. 1) so that it was suspended vertically. The ampoule was allowed to hang within a brass tube whose diameter was 1 inch. The electric motor was started so that the ampoule rotated rapidly about its own longitudinal axis, and the tumour tissue was flung in a thin even layer on to the sides of the bulb. While the ampoule was still spinning, a beaker of alcohol, brought to a temperature of below — 75° C. by the addition of carbon dioxide ice, was raised so that it surrounded the ampoule and its contents. In this way the

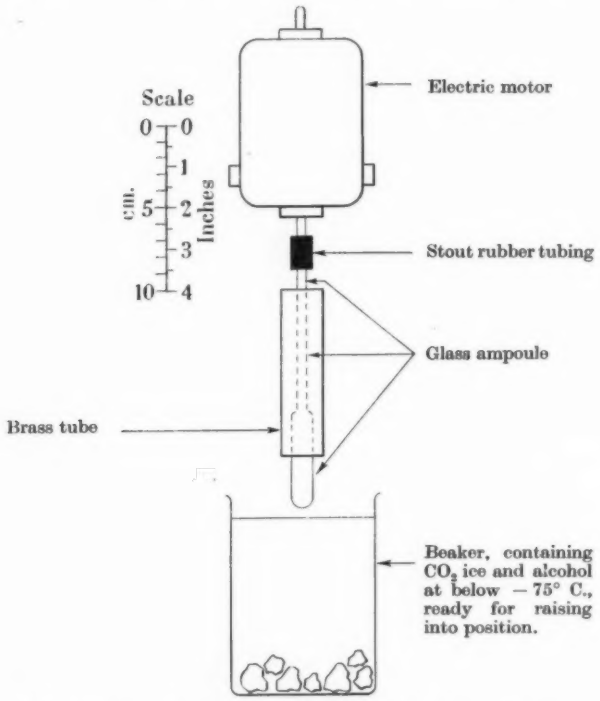

FIG. 1.—Diagram of shell-freezing apparatus for tumour mince.

tumour mince was frozen in a thin shell on the inside of the ampoule. After freezing, the ampoule and its contents were stored at a temperature of below — 75° C. in alcohol and carbon dioxide ice contained in a vacuum flask. Usually the tumour mince was kept at this temperature for 1 hour, and, unless it was to be freeze-dried, the contents thawed in running tap water at a temperature of about 20° C. It was then titrated as described above.

On those occasions when quick-freezing preceded freeze-drying by Method II (see below), then tumour mince was placed in test tubes (1 in. × 6 in.) fitted with a B. 29 ground glass socket (B.S.S.). The test tubes were spun about their longitudinal axes and frozen as described for the ampoules. The test tubes

were fitted with rubber bungs, in the upper surface of which holes were made to fit the spindle of the electric motor.

Slow freezing of sarcoma mince.

On some occasions tumour mince was placed in ampoules or test tubes 1 in. × 6 in. fitted with a ground glass socket (B.S.S., B. 29); these were spun about their longitudinal axis, as described under "Quick Freezing," in alcohol to which chips of carbon dioxide ice were added one by one, thus bringing the temperature down slowly, until the tumour mince was frozen. The tube or ampoule was then transferred to a thermos flask containing alcohol at the same temperature as that from which it was taken, and the slow freezing process continued until the temperature reached below — 75° C. This method was always used when slow freezing preceded freeze-drying, ampoules being used for Method I and test tubes for Method II (see below under the heading "Freeze-drying"). Because this method of slow-freezing was somewhat difficult in practice, on other occasions the mince was placed in a lump in ampoules or test tubes and the temperature brought down slowly as described above.

In both the methods of slow-freezing the temperature of the alcohol surrounding the test tubes or ampoules was taken at intervals. The temperature fell from between + 10° C. and + 20° C. to below — 75° C. over a period of time which ranged from 50 to 80 minutes in different experiments. The temperature fall is shown in Table I and Fig. 2. After the temperature had fallen below — 75° C. the tumour mince was allowed to remain in alcohol at the same temperature for a period of at least 1 hour. At the end of this time, unless it was to be freeze dried, it was thawed in running tap-water and titrated as described above.

TABLE I.—*Showing Temperatures at Five-minute Intervals During Five Slow Freezing Processes.*

Time in minutes.	Temperature of alcohol surrounding ampoules, °C., in Experiment—				
	51	110	139	149	166
0	17	20	17	16	20
5	3	15	13	11	15
10	−2	11	7	4	0
15	−7	5	−4	−3	−7
20	−10	0	−10	−10	−18
25	−15	−5	−24	−17	−23
30	−20	−10	−34	−26	−28
35	−25	−15	−40	−32	−42
40	−30	−20	−48	−40	−51
45	−35	−29	−55	−55	−61
50	−40	−37	−65	−65	−72
55	−48	−46	−76	−74	..
60	−50	−54	..	..	..
65	−60	−64	..	..	..
70	−66	−73	..	..	..
75	−71	−77	..	..	..
80	−77	..	..	..	..

This shows the temperature fall in 5 out of the 6 experiments in which slow freezing was carried out.

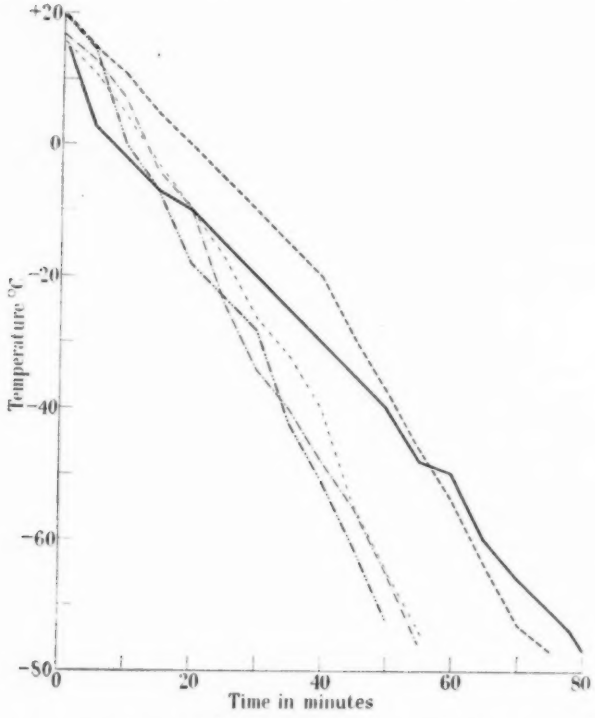

FIG. 2.—Graph of temperature fall in five slow-freezing experiments, showing temperatures at five-minute intervals.

Experiment 51 ————————
" 110 - - - - - - - - - -
" 139 —·—·—·—
" 149 — — — — — —
" 166 —··—··—··—

Freeze-drying of sarcoma tissue.

Freeze drying of sarcoma tissue was done by two methods. Apart from the device of freezing tumour mince in a shell by rapid spinning, described above, the method and apparatus used was fundamentally the same as that described by Craigie (1949*c*). In the first method used here a modification of the apparatus was made (Fig. 3), and in the second a modification of the method. The apparatus consisted of glass tubing and flasks whose bore was 1 in. or greater throughout. The different pieces were attached to one another by interchangeable ground-glass joints (B.S.S., B. 29). The apparatus was exhausted by a two-stage rotary pump (Model 2S 50, W. Edwards & Co.). The water-vapour was trapped by two condensers immersed in alcohol and carbon dioxide ice at a temperature of below — 75° C. The pressure within the apparatus was measured by a Pirani gauge (Model 5–2, W. Edwards & Co.) attached to the apparatus near

the pump. Craigie's (1949*c*) vapour flow indicator was not used. Diagrams of the apparatus are shown in Fig. 3 and 4.

Method I.—In this method, Flask No. 3 (Craigie, 1949*c*, Fig. 1) was replaced by a flask of the same dimensions whose neck was fitted with 8 small side arms, bore 6 mm., instead of a single large bore side arm (Fig. 3). These side arms were fitted with pieces of stout rubber tubing which, when the apparatus was not in use, were stoppered with pieces of glass rod of a suitable diameter. Immediately preceding use the apparatus was evacuated until the Pirani gauge showed a reading of between 0·005 and 0 mm. Hg. When the ampoules of tumour mince, frozen in a shell and kept at below − 75° C., were ready, the rubber tubing attached to the side-arms was clipped with Spencer Wells forceps

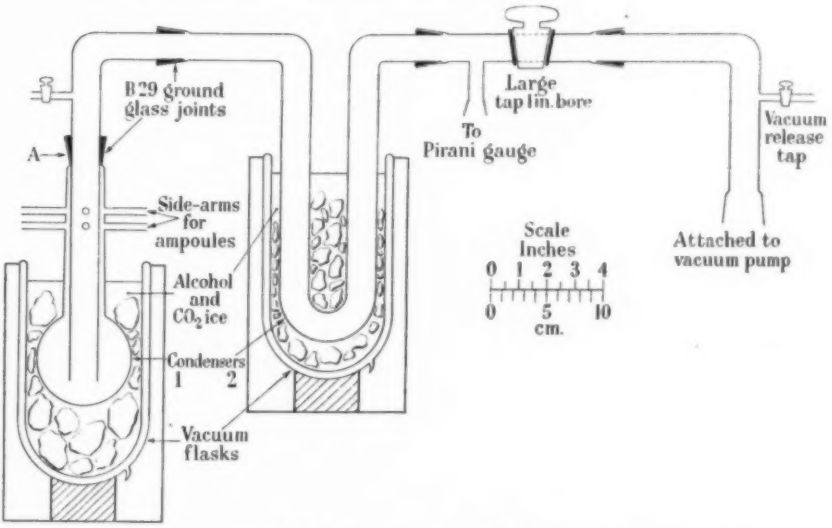

FIG. 3.—Diagram of all-glass freeze-drying apparatus for use with ampoules.

and the glass rod stoppers removed. The ampoules were quickly attached to the rubber tubing and the Spencer Wells forceps removed. At this point the pressure within the apparatus rose rapidly, but fell again to its previous level within 5 minutes, at which it remained throughout the drying period. Pumping was continued for at least 3 hours, after which the ampoules were removed, sealed and stored overnight at room temperature. The following day the ampoules were opened and 1 ml. of distilled water was added. This reconstituted tumour mince was then titrated as described above.

Method II.—In this second method, tumour material in quantities of 3 ml. or less was placed in 6 in. × 1 in. test tubes fitted with B. 29 (B.S.S.) ground glass sockets. The tubes were spun about their vertical axis and the tumour material frozen in a thin shell as described above. Sometimes the freezing was carried out slowly and sometimes quickly according to the experiment. The tubes were then attached to the freeze-drying apparatus in its original

unmodified form (Craigie, 1949*c*). The pump was allowed to run for at least 3 hours. During this time the tube containing the tumour mince was kept in an alcohol bath maintained at — 30° C. or below. No stirring mechanism was used; convection currents were relied upon to maintain an even temperature throughout the alcohol bath. At the end of the drying period the tube containing the dried tumour material was removed, lightly stoppered with cotton wool and placed in a desiccator over phosphorus pentoxide, where it was allowed to remain overnight. The following day a volume of distilled water was added equal to the original volume of material dried. The reconstituted material was then titrated.

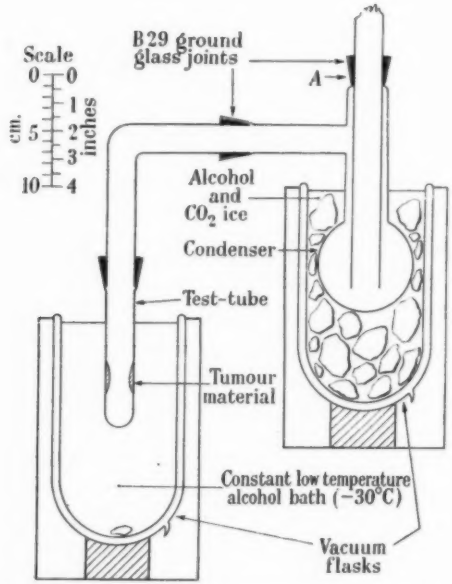

FIG. 4.—Attachment to freeze-drying apparatus for use with test-tubes.

Repeated freezing and thawing of sarcoma mince.

Quantities of mince, each 1 ml., were placed in ampoules, which were quickly frozen while spinning about their vertical axis as already described. The ampoules were placed in alcohol at a temperature of below — 75° C. for a period of 5 minutes, and then placed in running tap water at about 20° C. until the contents were thawed. This process was repeated until a total of 6 freezings and thawings had been completed. The resulting material was then titrated.

RESULTS.

Table II shows the results of slowly and quickly freezing sarcoma mince. This table shows in the first place that 37 sarcoma mince that had received no treatment whatsoever produced 100 tumours from 100 inoculations of 1

TABLE II.—*A Comparison of the Tumour-producing Activity of Untreated, Quickly Frozen and Slowly Frozen Minced Sarcoma 37.*

Experiment.	Material.	Dilution.					TPD50 index.	Result of culture of mince on blood agar.
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
41	Untreated	20/20	10/12	5/12	8/12	..	3·6	Micrococci ++
	Quickly frozen and stored 1 day	6/6	1/12	0/12	0/12	..	1·8	
	Quickly frozen and stored 16 days	9/10	..	..	..	..	—	
51	Untreated	20/20	12/12	5/12	1/8	..	3·1	Coliform bacilli +++
	Slowly frozen and stored 2 hours	6/6	6/12	0/12	0/8	..	2·2	
	Slowly frozen and stored 10 days	6/6	7/12	1/12	2/12	..	2·5	
110	Untreated	20/20	17/20	7/20	1/20	0/20	3·0	Sterile
	Quickly frozen	10/10	17/20	6/20	1/20	1/20	2·9	
	Slowly frozen	20/20	20/20	16/20	8/20	3/20	4·0	
139	Untreated	20/20	16/20	4/20	1/16	0/16	2·7	β-haemolytic streptococci ++
	Quickly frozen	15/15	10/20	0/20	0/20	1/20	2·2	
	Slowly frozen	20/20	10/12	1/20	..	..	2·6	
149	Untreated	20/20	20/20	2/20	5/20	2/20	2·9	β-haemolytic streptococci ++
	Quickly frozen	20/20	2/8	0/12	0/20	0/20	1·9	
	Slowly frozen	20/20	20/20	4/20	0/4	0/8	2·8	
166	Untreated	..	20/20	10/20	6/20	2/20	3·6	β-haemolytic streptococci +++
	Quickly frozen	20/20	8/20	1/20	2/20	0/20	2·2	
	Slowly frozen	20/20	12/20	2/20	0/20	0/20	2·5	
Totals each process from all experiments.	Untreated	100/100	95/104	33/104	22/96	4/76	3·1	
	Quickly frozen	80/81	38/80	7/84	3/92	2/80	2·3	
	Slowly frozen	92/92	75/96	24/104	10/64	3/48	2·8	

1. All the materials used in any one experiment were prepared from the same mince.
2. Inoculum of undiluted or diluted material was 0·06 ml. in every case.
3. Denominators show number of inoculations made.
4. Numerators show number of sarcomata resulting.
5. TPD50 index = x where the dose of mince which would be expected to cause sarcomata in 50 per cent of the mice is 10^{-x} ml.
6. .. = Not examined.

minim (0·06 ml.). With other unreported experiments the total number of 0·06 ml. doses of undiluted untreated mince was 612. From these 608 sarcomata resulted. Of the 4 mice in which no growth appeared, 2 were killed on the 7th and 2 on the 13th day because of intercurrent illness. All the tumours arose within 14 days as judged by the appearance of a progressively enlarging lump. The results of freezing Sarcoma 37 mince shown in Table II demonstrate clearly that, whether the mince had been frozen rapidly or slowly, its tumour-producing activity was not entirely destroyed. In fact undiluted mince treated in either way produced tumours in 172 cases out of 173—the one failure occurring in mince which had been frozen quickly and stored for 16 days (Table II, Experiment 41). The results of titrations show that in 3 cases (Experiments 51, 139, 149) out of 5 the slowly frozen mince was slightly less active than the control untreated material. In one case (Experiment 110) it was more active, and in the other (Experiment 116) it was considerably less active than the control material. In every experiment, however, the quickly frozen mince was less active than both the slowly frozen material (Experiments 110, 139, 149, 166) and the untreated control material (Experiments 41, 110, 139, 149, 166). The total figures for all experiments show that the TPD50 index of untreated material was 3·1, of quickly frozen material was 2·3, and the slowly frozen material 2·8. On two occasions (Experiments 41, 51) frozen mince was stored. In Experiment 41 the mince was frozen quickly and was stored for 16 days. No titration was performed on this material, but 9 tumours arose from 10 inoculations. In Experiment 51 the material was slowly frozen and stored for 10 days—the TPD50 index of this material was slightly greater than that of the same material which had only been stored for 2 hours.

All tumours which arose after freezing, slow or quick, appeared within 14 days, but did so in a slightly longer time than those arising from the corresponding untreated material.

Table II also shows the results of sterility tests. Only on one occasion was the mince found to be sterile (Experiment 110). In the first two experiments (Experiments 41, 51) the tests revealed contamination with micrococci in one and a coliform organism in the other. In the last three experiments (Experiments 139, 149, 166) the mince was contaminated with a β-haemolytic streptococcus. Although the minces were contaminated on each occasion all mice inoculated appeared to be perfectly fit and healthy, and the death rate among them was no greater than that occurring in the stock mice from which they were taken. Pathogenicity tests on mice were performed with the β-haemolytic streptococcus with negative results. Attempts to group the streptococcus have failed. Many attempts by various methods, including the use of antibiotics, have been made to free the mince from bacterial contamination without success.

Table III shows the result of inoculating tumour material which had been repeatedly (6 times) frozen and thawed. Four experiments were performed in this group. A total of 54 inoculations of frozen and thawed material was made. On no occasion did a progressively growing tumour arise. All mice were observed for a period of 35 days, and some of them were examined for longer periods up to 70 days. In the first two experiments (Experiments 8, 31) every single mouse inoculated with untreated control material developed tumours within 14 days. In the last two (Experiments 125, 184) no control

experiments were performed. This was thought to be of no great importance, since past experience had shown that undiluted untreated material produced tumours in at least 99·3 per cent of cases (608 tumours from 612 inoculations).

TABLE III.—*Showing the Effect of Freezing and Thawing Six Times on the Tumour-producing Activity of Minced Sarcoma 37.*

Experiment.	Tests.			Controls.		Result of culture of untreated mince on blood agar
	Number of mice.	Number of inocula.	Number of tumours.	Number of days observed.	Number of inocula.	Number of tumours.	
8	5	5	0	36	50	50	Coliform bacilli +++
31	3	3	0	36	20	20	Sterile
125	30	30	0	35–52	..	..	,,
184	8	16	0	38–70	..	..	Micrococci +
Totals	46	54	0	..	70	70	

Table IV shows the results of freeze-drying experiments. Of a total of 113 inoculations of freeze-dried material, not a single progressively growing tumour arose. In the first 5 experiments the material was frozen rapidly before drying, and in the last 5 it was frozen slowly. In Experiments 110*a*, 110*b* the material used was derived from the same tumour mince. However, slow freezing before freeze-drying did not affect the results. Except for one experiment (Experiment 149*a*), in which the mice died from an accident, all mice were examined for more than 36 days, mostly for longer periods up to 107 days (Experiment 139*a*). Out of the 10 experiments, all but 1 (Experiment 185) were performed in parallel with control observations made on untreated material from the same source. The results of sterility tests of the experiments summarized in Tables III and IV are shown in the appropriate place. On 4 occasions (Table III, Experiments 31, 125; Table IV, Experiments 110*a*, 110*b*) a sterile mince was obtained. On the other occasions the minces were contaminated to some degree or other.

In all the experiments summarized in Tables III and IV, although no progressively growing tumours were obtained, the inoculated material could be felt as a subcutaneous nodule during the first few days. These nodules gradually disappeared. In experiments not reported here, histological examination of such inocula of freeze-dried mince up to the 7th day revealed no evidence of tumour growth.

DISCUSSION.

The results of the work reported here show, firstly, that freezing of Sarcoma 37 tissue does not deprive it of its tumour-producing activity, and that this property is less affected by slow freezing than by quick freezing (Table II). This finding is in agreement with those of Breedis, Barnes and Furth (1937) and Breedis and Furth (1938) who worked with leukaemic cells. Secondly, the results reported here show that repeated freezing and thawing on 6 occasions or drying from the frozen state deprived Sarcoma 37 tissue of all its tumour-producing activity.

One criticism that can be made of this work is that on most occasions the tumour minces investigated here were contaminated, to a greater or less degree,

TABLE IV.—*Showing the Effect of Freeze-drying on the Tumour-producing Activity of Minced Sarcoma* 37.

Experiment.	Tests. Method of freezing.	Method of drying.	Number of mice.	Number of inocula.	Number of tumours.	Number of days observed.	Controls. Number of inocula.	Number of tumours.	Result of culture of untreated mince on blood agar.
8*a*	Quick	I	4	4	0	36	50	50	Coliform bacilli +++
8*b*	,,	I	3	3	0	43			
41	,,	I	5	10	0	56	20	20	Micrococci ++
58	,,	I	5	10	0	59	20	19	Micrococci +
110*a*	,,	II	5	10	0	39	20	20	Sterile
110*b*	Slow	II	5	10	0	39			
139*a*	,,	II	4	16	0	107	20	20	β-haemolytic streptococci ++
149*a*	,,	II	5	20	0	10	20	20	β-haemolytic streptococci ++
166*a*	,,	II	6	12	0	75–82	20	20	β-haemolytic streptococci +++
185	,,	II	9	18	0	70	..	..	Scanty micrococci
Totals	..	..	51	113	0	..	170	169	

TABLE V.—*Showing the Effect of Storage at* − 79° *C. on the Transplantation of Two Mouse Carcinomata*: (*Compiled from the Results of Mann*, 1949b, *Table I.*)

Tumour material was minced and then stored at − 79° C. for various periods of time. The stored material was then inoculated into mice and the number of tumours resulting were recorded.

	Tumour "63"			Sporadic tumour.			Both tumours.		
	Inoculations.	Tumours.	Per cent tumours/inoculations.	Inoculations.	Tumours.	Per cent tumours/inoculations.	Inoculations.	Tumours.	Per cent tumours/inoculations.
Storage for 48 hrs. and less	84	37	44	38	35	92	122	72	59
Storage for more than 48 hrs.	24	12	50	79	70	89	103	82	80
Totals	108	49	45	117	105	90	225	154	69

with bacteria (Tables II, III, IV). However, in most of the experiments on freezing and repeated freezing and thawing such tumour material was kept for only a short period of time before reinoculation, and during that time it was maintained at a temperature of below $-75°$ C. The mice into which the contaminated tumour material was inoculated showed no signs of constitutional disturbance, and in most cases adequate control inoculations were made of material from the same source as that used in each experiment. In these cases it was felt that bacterial contamination was of no significance. In the case of the freeze-drying experiments (Table IV) material was kept in the dried state for 24 hours before reconstitution and reinoculation. Under these conditions it is just conceivable that the products of bacterial metabolism might have an effect on the tumour-producing activity of the freeze-dried material. However, this is thought to be unlikely, especially in those experiments where the tumour material was subjected to drying, primary and secondary throughout the 24 hours by Method II of freeze-drying. Every attempt was made to eliminate bacterial infection, and it is obviously desirable to do so. However, provided that the degree of infection is noted, it does not necessarily detract from the value of the observations.

The quantitative method described in this paper was chosen rather than one based on the time of first appearance of tumours, for the reason that the time of first appearance of a tumour was difficult to determine. The original inoculum of tumour material could, in nearly every case, be felt immediately after inoculation. Every inoculum showed a slight initial enlargement, even if it had been made with material which had been inactivated by repeated freezing and thawing or freeze-drying. This enlargement was shown by histological methods to be due to an inflammatory reaction which on occasions was not due to bacteria, because it occurred with samples of material which were bacteriologically sterile. Thus, it was impossible to know, by palpation, where inflammatory reaction ended and sarcoma growth began. Initially, a quantitative method based on the rate of growth of tumours as judged by caliper measurements was tried. This was found to be subject to immense variation even when a large number of observations were performed. Thus the only practicable method available was one such as is described here. It has no pretensions of great accuracy, because it is technically difficult to obtain a dispersion of tumour mince to the same degree as can be obtained with viruses. It is claimed, however, that the method described here when used on a sufficiently large scale is more accurate than those used heretofore.

It is to be noted that tumour mince was diluted immediately before titration. All treatments used in this work were applied to undiluted mince, and thus the variable effect of diluents has been entirely eliminated. Also the technique of freezing tumour mince in a thin shell in standard-sized ampoules or test tubes has ensured that exposure to cold or drying processes has been as nearly strictly comparable as possible. Only in the case of slow freezing was tumour tissue exposed to cold in a lump, but here the only purpose was to ensure that the temperature of the tumour mince fell slowly. The fact that the temperature of some parts of the mince may have fallen at a slower rate than others was not of great consequence, because the purpose of the experiment was to distinguish between quickly frozen material and slowly frozen material, and not to draw conclusions, at this point, about the effect of the rate of fall of temperature.

The effect of cold on Sarcoma 37 has not been widely studied. Gye, Begg, Mann and Craigie (1949) used this tumour in one experiment, where it was stored without diluent at — 78° C. for a period of 201 days. Five inoculations of this frozen and stored material produced 5 tumours in 7 days. This is not at variance with the findings reported here (Table II). Passey and Dmochowski (1950) report similar results.

Cramer (1930) was successful in obtaining tumours from grafts of material which had been repeatedly frozen and thawed either 4 or 8 times. This differs from the findings here (Table III); however, Cramer's technique differed from that used here. In one experiment Cramer exposed his material to a temperature of — 20° C. In the other he used a temperature of at least — 80° C., but exposed his tumour material to it in a mass contained in a test tube. Under this condition the destructive effect of repeated freezing and thawing may have been diminished. The difference in technique may account for the conflicting results. In addition it is possible that the properties of Sarcoma 37 vary from time to time, and that the strain of mice used may affect the outcome of experiments with this tumour.

Mider and Morton (1939) also using 37 stated that they were able to obtain tumours in 10 per cent of cases after freezing and thawing the tumour tissue *en bloc* 4 times. The number of mice used in their experiments is not stated. Selbie and McIntosh (1939) were unable to obtain growth from Sarcoma 37 tissue which had been repeatedly frozen and thawed.

Mider and Morton (1939) dried Sarcoma 37 from the frozen state and failed to obtain tumours from the dried material. However, Passey and Dmochowski (1950) were able to obtain growth from dried material of the same tumour desiccated in 5·3 per cent glucose solution. The use of the glucose solution may account for the fact that these findings are at variance with those reported here; no diluent was used during desiccation in the work reported in the present paper.

The first method (Method I) of freeze-drying used here was less efficient than that used by Gye, Begg, Mann and Craigie (1949), but the second method was more so. Firstly, because the tumour mince was spread thinly and evenly over the wall of the large test-tubes, and secondly, because secondary drying over phosphorus pentoxide was carried out. Despite this added efficiency no tumours were obtained.

It should be noted that none of the tumour mince used here was stored in the frozen state for any length of time before freeze-drying; this is of importance, because Gye, Begg, Mann and Craigie (1949) state that they obtained a greater number of tumours with material which had been stored at — 78° C. for some time before freeze-drying. There is no evidence provided for this statement, but Mann (1949*b*) says that tumour material which had been stored at — 78° C. for longer than 48 hours produced more tumours than that which had been stored for a shorter period. In view of the implications of this statement it is thought worth while to review the details of her results. If Mann's tabulated results (Mann, 1949*b*, Table I) are examined, it will be noted that she used two different tumours, " 63 " and " Sporadic." Her results, excluding those experiments (M60A, M65 and M72) in which technical errors occurred and which she herself excluded in her analysis, are re-tabulated in Table V in such a way that each tumour is considered separately. It will be seen that the total incidence

of tumours derived from " 63 " frozen material, stored for whatever length of time, is 45 per cent (49 tumours from 108 inoculations), and for those observations made on tumour material stored for less than 48 hours it is 44 per cent (37 tumours from 84 inoculations). For those observations made on tumour material stored for more than 48 hours the incidence of tumours is 50 per cent (12 tumours from 24 inoculations). The difference between the percentage incidences before and after 48 hours' storage (44 per cent and 50 per cent respectively) is not statistically significant (Standard Error of difference = 11·5 per cent). Similarly, with the " Sporadic " tumour: the percentage incidence for all frozen Sporadic material is 90 per cent (105 tumours from 117 inoculations), that for material stored for less than 48 hours is 92 per cent (35 tumours from 38 inoculations), and that stored for more than 48 hours is 89 per cent (70 tumours from 79 inoculations). Again and more obviously the difference between the incidences before and after 48 hours' storage is not significant (Standard Error of the difference = 5·6 per cent).

Further examination of Mann's (1949*b*) table shows that in each case the incidence of tumours resulting from the inoculation of frozen and stored material is approximately the same for whatever period of time storage was carried out. In the case of tumour " 63 " this will be 45 $\pm$ 9·6 per cent, and for " Sporadic " it will be 90 $\pm$ 5·6 per cent. If account is taken of this and the fact that in the first 48 hours most of Mann's (1949*b*) observations (84 out of a total of 122) were made on tumour " 63," and after 48 hours most observations (79 out of 103) were made on the " Sporadic " tumour, then her conclusions are explained. Clearly, the conclusions are based on an initial bias in the form of an unequal weighting of material which vitiates subsequent statistical analysis.

It was considered important to deal with this matter is detail here for four reasons :

1. Because the statement arising from the results as presented by Mann (1949*b*) is misleading.
2. The results, as interpreted here, suggest that the storage of a particular tumour at — 79° C. for any period of time up to 182 days does not greatly affect the tumour-producing activity of the material.
3. The results, as interpreted here, suggest the possibility that freezing to low temperatures affects the tumour-producing activities of different tumours to different degrees.
4. From " 2 " it may follow that freezing to — 79° C. is a convenient method of storing standard samples of tumour material.

As a result of the above it was considered that there was no reason to believe that there was necessarily an advantage in storing tumour material below — 75° C. before desiccation. Therefore this practice was not adopted in the present investigation. However, the figures shown in Table I suggest that there would be an advantage in slowly freezing Sarcoma 37 material before freeze-drying. This is based on the assumption that the more active the starting material the more likely it would be to survive the freeze-drying process. However, this manoeuvre failed to produce material which was active after freeze-drying (Table IV).

Whether or not the induction of tumours by neoplastic tissue which has been frozen or dried implies causation by virus depends upon whether the treat-

ment applied has killed the cells of the particular tumour in question. This same argument would apply to any treatment, such as with glycerol, to which tumour cells may be subjected in an attempt to kill them and liberate virus.

Hirschberg and Rusch (1950) quote evidence that some normal tissues have survived freezing to low temperatures and have grown subsequently, whereas other normal tissues will not. Klinke (1940) froze different types of tumour material, and obtained growth subsequently, in tissue culture, of some types of tumour but not others.

The effect of drying on the viability of mammalian cells has been little studied : spermatozoa have retained their motility after drying (Polge, Smith and Parkes, 1949). Passey and Dmochowski (1950) have obtained growth of 4 mouse sarcomata, including 37, after freeze-drying, in one case in tissue culture (Passey, Dmochowski, Lasnitski and Millard, 1950). On the other hand, Mider and Morton (1939) failed to obtain growth of 37 after freeze-drying.

Although differences in technique may account for some of the apparently conflicting findings it does not explain them all. It can be seen that it is probable that some types of mammalian cell, normal or neoplastic, will survive freezing and drying whereas others will not. The tissue culture work is particularly cogent. Cells must be alive, whatever treatment may have been applied to them previously, when they grow in tissue culture. On the other hand, if no growth of treated cells is obtained in tissue culture, it does not mean that such cells are not viable. Growth may not be obtained in the artificial environment of tissue culture (or, for that matter, in any unusual site *in vivo*) with cells whose activity has been depressed by some treatment or other ; but there is no reason why it should not occur under optimal conditions at usual sites *in vivo*. This argument can be applied to the findings of Mann (1949*b*), who inoculated frozen tumour material subcutaneously and obtained tumours, but failed to do so when the inoculations were made intraperitoneally. Thus, a negative result in tissue culture or in any unusual site *in vivo* and a positive one in a usual site *in vivo* is not proof of the presence of virus.

In the recent work by Gye and his colleagues (Gye, Begg, Mann and Craigie, 1949) no attempt was made to see if the particular tumour cells being used survived the treatments applied to them. All the results, recent and remote, can be explained by the survival of cells, and there is evidence that some types of cell survive freezing and drying. There is no justification, on the evidence provided, to invoke a virus ; although a virus is not absolutely excluded.

If a virus, as we know it, were present in the tumours and were released by these treatments applied to it, then it should be demonstrable. Passey and Dmochowski (1950) failed to demonstrate virus in saline extracts of frozen and dried tumours, including 37. Thus, the evidence is against a virus aetiology.

Assuming, then, that tumour cells do survive in these cases, then inocula of frozen Sarcoma 37 material should show tumour cells when examined histologically at various intervals after inoculation. Similarly, inocula of repeatedly frozen and thawed or freeze-dried material should show no such cells. An investigation along these lines has been carried out, and will be reported in a subsequent paper. Such methods together with those described in this paper if applied to other tumours should reveal useful information which will go towards settling problems arising in this field. Also, the findings are of value in them-

selves, in that they show methods which will enable samples of tumour tissue of known potency to be stored for use when required.

SUMMARY.

A quantitative method has been applied to the study of Sarcoma 37 of mice. The method consists of the determination of the dose of tumour mince which will produce sarcomata in 50 per cent of mice. The method was applied to Sarcoma 37 after freezing to below — 75° C., repeatedly freezing and thawing and freeze-drying.

The technique of freeze-drying is that of Craigie (1949*c*), slightly modified. The modifications are described.

The results show that freezing quickly to below — 75° C. depresses the tumour-producing activity of 37 material to a greater extent than freezing slowly to the same temperature. Also it is shown that freeze-drying and repeatedly freezing and thawing entirely destroy the tumour-producing activity of Sarcoma 37 mince. These last two findings are in conflict with other reports.

The technique used and the results are discussed in detail.

Certain aspects of the reports of other workers in the same field are also discussed in detail. It is concluded that the evidence is against the release of viruses from tumours by freezing and drying techniques. A suggestion is made for the useful application of the work described.

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THE FATE OF GRAFTS OF SARCOMA 37 MINCE AFTER EXPOSURE TO LOW TEMPERATURE AND FREEZE-DRYING.

P. T. J. C. P. WARNER, J. V. T. GOSTLING AND A. C. THACKRAY.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W.1.

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NUMEROUS workers have subjected normal and neoplastic tissues to low temperatures and some to drying. Many of the reports of these workers have been referred to in a previous paper (Warner and Gostling, 1950).

Some workers have assumed or suggested that freezing and freeze-drying processes killed the cells that they were studying (Salvin-Moore and Walker, 1908; Salvin-Moore and Barratt, 1908; Gaylord, 1908; Rous, 1913; Cramer, 1930; Cramer and Foulds, 1930; Gye, 1949; Gye, Begg, Mann and Craigie, 1949; Man, 1949*a*, 1949*b*; Mann and Dunn, 1949). However, most of these workers made no attempt to determine directly whether the actual cells that they exposed to low temperatures and freeze-drying survived or not.

On the other hand, Mider and Morton (1939), using rat skin, Blumenthal and Walsh (1950), using guinea-pig thyroid, and Dmochowski and Millard (1950), using C3H and C48 mouse sarcomata, subjected tissue to low temperatures or freeze-drying and studied their behaviour, by histological methods, when grafted into animals. These workers found evidence of growth or survival of cells and mitotic activity in the grafted tissue.

Other workers have subjected tissues to low temperatures or freeze-drying and subsequently attempted to grow them in tissue culture (Cramer, 1930; Klinke, 1940; Passey, Dmochowski, Lasnitski and Millard, 1950). The results showed survival of some types of tissue, neoplastic and normal, and not of others.

In tissue culture, Cramer (1930) obtained no growth of Sarcoma 37 which previously had been repeatedly frozen and thawed, whereas grafts of the same tissue grew *in vivo*. Passey and Dmochowski (1950) dried a suspension, in glucose solution, of minced Sarcoma 37, from the frozen state. More glucose solution was added to the dried material and it was then inoculated into mice. Five out of the ten mice thus inoculated developed tumours.

A previous paper (Warner and Gostling, 1950) described experiments in which minced Sarcoma 37 which had been frozen, quickly or slowly, to a temperature of below — 75° C. was capable of producing tumours when inoculated into mice. It was also shown, contrary to Cramer (1930) and Passey and Dmochowski (1950), that if the tissue were repeatedly frozen and thawed or dried from the frozen state, no tumours resulted.

Since some workers have assumed that freezing or freeze-drying kills tumour cells and that a virus must be implicated in the transmission of tumours by

tissue treated in such a way, it was considered important to study the fate of grafts of Sarcoma 37 under similar conditions.

This paper describes the results of histological examinations, at various intervals after inoculation, of grafts of Sarcoma 37 untreated and previously treated by slow freezing, quick freezing (to below −75° C. in each case) and drying from the frozen state.

MATERIALS AND METHODS.

The material and methods used for preparing Sarcoma 37 mince, and for freezing, repeatedly freezing and thawing and freeze-drying it have been described in detail in a previous paper (Warner and Gostling, 1950) and will be briefly recapitulated here. The sarcomata were grown in albino mice, removed from them and minced in a Craigie pressure mincer (Craigie, 1949*a*). The mince was tested for bacteriological sterility. It was then either left untreated and inoculated into mice as described below, or was subjected to slow or quick freezing or repeated freezing and thawing (six times), in each case reaching a temperature of − 75° C., or dried from the frozen state by Method II (Warner and Gostling, 1950). This method of freeze-drying is practically the same as that described by Craigie (1949*b*), except that in addition secondary drying over phosphorus pentoxide was performed. Some of each of the untreated mince, that frozen by the slow and quick methods and that frozen and thawed repeatedly, was examined histologically ; the remainder was then inoculated, in doses of 1 minim, into both flanks of each one of five or more mice. The mince that had been dried from the frozen state was reconstituted by adding a volume of distilled water equal to the original volume of tumour mince subjected to the drying process. This reconstituted material was then inoculated into the flanks of mice in doses of 1 minim, exactly as described above.

At various intervals, ranging from 3 hours to 10 days, one or some of the mice were killed. The grafts were removed, fixed in Helly's fluid, histological sections were prepared and stained with haematoxylin and eosin. In some cases serial sections were prepared from the grafts.

RESULTS.

The results are shown partly in Table I and Fig. 1 to 6 and 8 to 13, and are summarized in Fig. 7. The bacteriological sterility tests showed that the minces used in Experiments 198, 199*a*, 199*b*, 199*c*, 220, 221 and 222 were contaminated with β-haemolytic streptococci which were encountered in previous work (Warner and Gostling, 1950). A few colonies of micrococci were grown from the minces used in Experiments 184 and 185. However, there was no histological evidence of gross bacterial inflammatory processes.

The photographs in Fig. 1 to 6 show the histological appearances of the tumour mince before treatment (Fig. 2) and after slow freezing (Fig. 3), quick freezing (Fig. 4), repeatedly (× 6) freezing and thawing (Fig. 5) and after freeze-drying and reconstitution by the addition of distilled water (Fig. 6). It will be seen that the appearances of the nuclei of the minces treated by any of the methods described here differ from those of the original tumour. The nuclei are more densely staining and slightly shrunken. The differences between the minces are small (Fig. 2 to 6), except perhaps in the case of that which had been

repeatedly frozen and thawed (Fig. 5), where the nuclei are even more dense and shrunken than the others. Otherwise it is difficult to distinguish between them by histological methods.

TABLE I.—*Incidence of "T" Cells in Peripheral Rim of Grafts and Onset of Frank Tumour Growth.*

Treatment of mince.	Experiment.		Time after inoculation. Hours. 3.	6.	12.	Days. 1.	2.	3.	4.	5.	6.
None	198	"T" cells	..	..	..	+++	+++	++	..	±	..
		Tumour	..	..	..	−	−	−	..	T	..
	199a	"T" cells	−	+	++	..	..	..	..	..	..
		Tumour	−	−	−	..	..	..	..	..	..
	220	"T" cells	..	..	..	+++	+++	++	+	+	±
		Tumour	..	..	..	−	−	−	T	T	T
Slowly frozen	221	"T" cells	−	+	+	++	++	++	−	±	−
		Tumour	−	−	−	−	−	−	−	−	T
Quickly frozen	222	"T" cells	..	..	..	±	±	±	−	±	−
		Tumour	..	..	..	−	−	−	−	−	T
Repeatedly frozen and thawed	184	"T" cells	..	..	..	−	−	−	−	−	..
		Tumour	..	..	..	−	−	−	−	−	..
	199b	"T" cells	−	−	−	..	..	..	..	..	..
		Tumour	−	−	−	..	..	..	..	..	..
Freeze-dried	185	"T" cells	..	..	..	−	−	−	−	−	−
		Tumour	..	..	..	−	−	−	−	−	−
	199c	"T" cells	−	−	−	..	..	..	..	..	..
		Tumour	−	−	−	..	..	..	..	..	..

+++ = Numerous
++ = 5–15 per section
+ = 1–5 „ „
± = 1 „ „
± = 1 in 5 or more sections
} "T" cells.
T = Frank tumour growth.
.. = Not examined.

Results of histological examination of the various types of graft are shown in Table I, and are summarized in Fig. 7. The following six points were considered in each section :

1. Necrosis as judged by the disappearance of nuclei from sections of grafts.
2. Polymorphonuclear leucocyte invasion of the grafts.
3. Fibroblast and capillary invasion of the graft from surrounding tissue.
4. The presence of certain characteristic cells which are believed to be, in all probability, single tumour cells, but here are non-committally called "T" cells.
5. Mitotic activity.
6. Frank tumour growth.

Certain changes, namely necrosis, polymorphonuclear leucocyte invasion and fibroblast and capillary invasion, were more or less the same for every type of graft, untreated, frozen quickly or slowly, repeatedly frozen and thawed and freeze-dried. These changes will be described together and are summarized in Fig. 7.

Necrosis was noticed in the earliest sections of grafts removed 3 hours after inoculation. It started at the periphery of the graft and in grafts removed after

longer periods of time it moved towards the centre until finally the graft consisted almost entirely of necrotic tissue. While necrosis was proceeding polymorphonuclear leucocytes invaded the graft in ever increasing quantities, reaching a maximum about the second day. Thereafter the number of polymorphs declined, first at the periphery, leaving a rim of necrotic tissue where either a few or no cells could be seen. After the first day, in the tissues surrounding the graft, capillaries appeared, and fibroblasts which later formed a capsule and eventually began to invade the graft itself, singly and in finger-like processes together with capillaries. The above findings were common to all grafts. Fig. 8 shows the appearance of a section at one stage of this process.

Examination of sections of grafts of untreated material showed that at 3 hours most cells had become round, the cytoplasm stained a dense pink and the nuclei were pyknotic. Occasionally a few normally staining cells were seen. At 6 hours there were many ghost cells to be seen and the polymorphonuclear invasion had started. After one day, among the necrotic debris, within the peripheral rim of the graft, there were large cells with large nuclei and a fair amount of slightly basophilic cytoplasm (Fig. 9); these cells were irregular in outline. The nuclei were rounded or irregular in shape. At first they were seen in the peripheral rim of the graft, but not outside it. Then, during the first and second day, these characteristic cells seemed to become larger and more irregular in outline, then there appeared similar cells in the gap between the graft and the surrounding tissues (Fig. 9) and in the surrounding tissues themselves. Collections of similar cells could be seen near capillaries in the tissues surrounding the graft (Fig. 10). At about this time (3 days after inoculation) these basophilic cells with large nuclei, described above, appeared to diminish in number in the peripheral rim of the graft. On the fourth day frank tumour appeared, not within the graft, but in the tissues surrounding it, and the cells constituting the early tumour were similar to those which first appeared within the peripheral rim of the graft and then between the graft and surrounding tissues. Again, these characteristic cells were seen within the peripheral rim of the graft near early tumour growth and also at some distance from the tumour but in much smaller numbers than were seen in earlier stages. These large cells were thought to be tumour cells and were called, non-committally, " T " cells. A search was then made for " T " cells in the sections of grafts of mince which had previously been exposed to low temperatures and freeze-drying.

The grafts of material which had been slowly frozen showed the same train of events as had the untreated material, except that typical " T " cells were much scantier. After examination of serial sections no " T " cells were found in a graft removed 4 days after inoculation, and only 2 such cells in a graft removed 5 days after inoculation. " T " cells appeared slightly less " healthy " than similar cells in grafts of untreated mince. Fig. 11 and 12 show their appearance in a graft of slowly frozen mince removed 2 days after inoculation. Characteristic " T " cells are to be seen in the graft itself and one beyond the edge (Fig. 11), apparently applying itself to the surrounding tissues. With the exception of the fourth day, " T " cells could be detected within the graft until frank tumour appeared on the sixth day.

In grafts of quickly frozen material the process was very difficult to follow. " T " cells were very scanty within the graft and characteristic ones were very rare. However, they could be detected on the first, second and third days, and

tumour was found on the fifth day. Again, the characteristic properties of these cells were slightly less well defined than in grafts of slowly frozen mince.

So, the " T " cells, which there is reason to consider are viable because they retain their staining characteristics when all other cells have necrosed, and which there is reason to believe are tumour cells, are found most numerously in grafts of untreated tumour mince, less so in slowly frozen, and again less in quickly frozen. Thus, their number is proportional to the tumour-producing activity of the three preparations as determined on a previous occasion (Warner and Gostling, 1950), and as shown in Fig. 7.

Examination of the repeatedly frozen and thawed and freeze-dried material presented some difficulty; for although no typical " T " cells were found when serial sections of grafts removed after the first day were examined, it was possible to find cells in earlier sections which showed some resemblance to them, but usually these were small and their nuclei pyknotic. Had such cells been seen in grafts of untreated or slowly or quickly frozen material they would not have been accepted as reaching the criteria by which " T " cells were defined. Such cells as were seen were few and far between.

The fifth point which was considered in the examination of sections of grafts was mitotic activity. Mitotic figures were seen in the connective tissue surrounding the grafts of all material treated and untreated removed after 2 days. When serial sections were examined approximately one mitosis was found in each section. In grafts removed later (4 days after inoculation) mitoses in the surrounding connective tissue were much less frequent. The mitoses were regular and symmetrical and lacked the massive and heavily-stained appearance of those seen in fully-developed tumours.

No mitoses were seen in characteristic " T " cells nor were any mitoses seen within the grafts of untreated or slowly or quickly frozen material. Mitoses within grafts were seen only in 5 sections of repeatedly frozen and thawed and freeze-dried material. In 3 of these (repeatedly frozen and thawed material after 2 days and 5 days, and freeze-dried material after 3 days) the figures had the same symmetrical appearance as those previously described in surrounding connective tissue. The fourth and fifth (repeatedly frozen and thawed after 3 days) showed two mitoses in cells with irregular outlines. One is shown in Fig. 13 and, though by no means characteristic, bore a possible resemblance to a " T " cell.

DISCUSSION.

It seems clear from the results described here that the fate of a graft of untreated Sarcoma 37 mince is as follows: at first necrosis of most of the cells takes place. A few (" T " cells), however, remain viable, enlarge and move to the edge of the graft (Fig. 9) and thence into the surrounding tissues of the host where nutriment from nearby capillaries is available (Fig. 10). Having reached a favourable environment the " T " cells multiply and develop into frank tumour. The hypothesis of migration of tumour cells is borne out by the work of Coman (1947) and McCutcheon, Coman and Moore (1948), who showed that tumour cells are less sticky and more motile than normal cells. Other features recorded here, such as the necrosis of the majority of cells in a graft, the polymorph invasion, the fibroblast and capillary multiplication and the survival of occasional cells near the edge of grafts have been described in detail by workers from the

time of Loeb (1901, 1902), Jensen (1903) and Murray and Bashford (1904, 1905). The fact that tumour cells do migrate would explain the finding that tumour growth occurs first not within the graft itself but in the tissue around it. It is not necessary to postulate the presence of a virus to account for the site of tumour growth.

The number of " T " cells found in minces which previously had either been

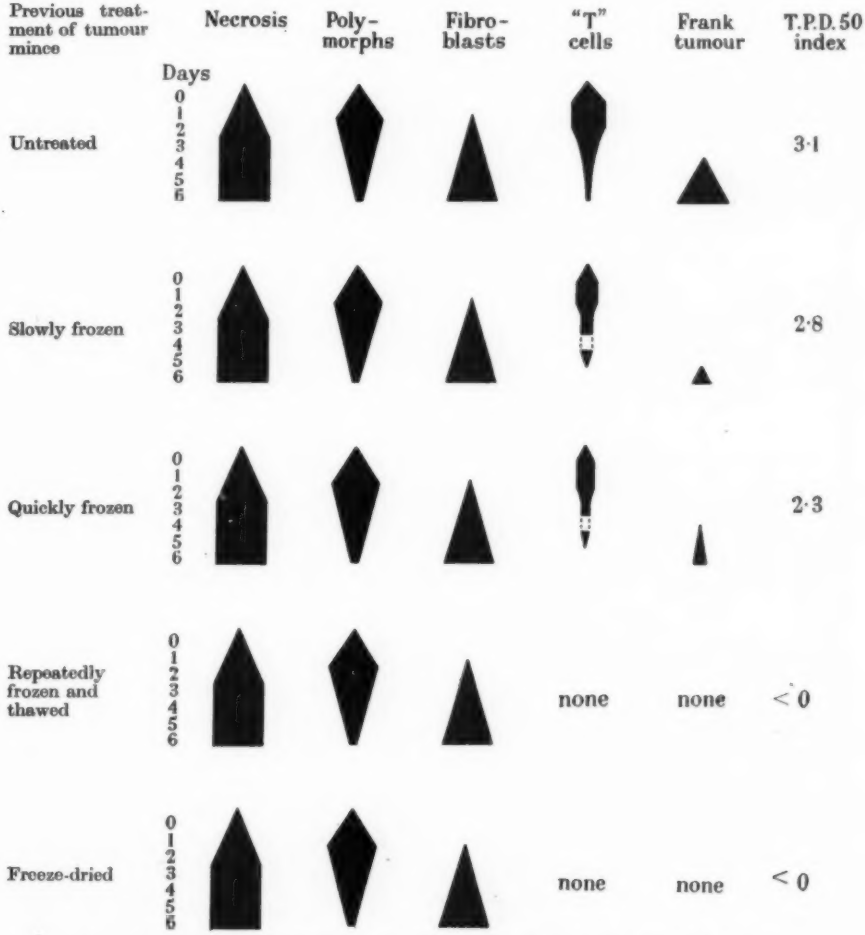


FIG. 7.—Diagrammatic representation of the fate of grafts of Sarcoma 37 mince.

In each vertical column the transverse measurement of the black area is roughly proportional to the degree of the process indicated at the head of the column. The measurements in one vertical column are not to be compared with those in another.

T.P.D.50 index = negative logarithm of the volume of tumour mince required to produce tumours in 50% of mice (Warner and Gostling, 1950).

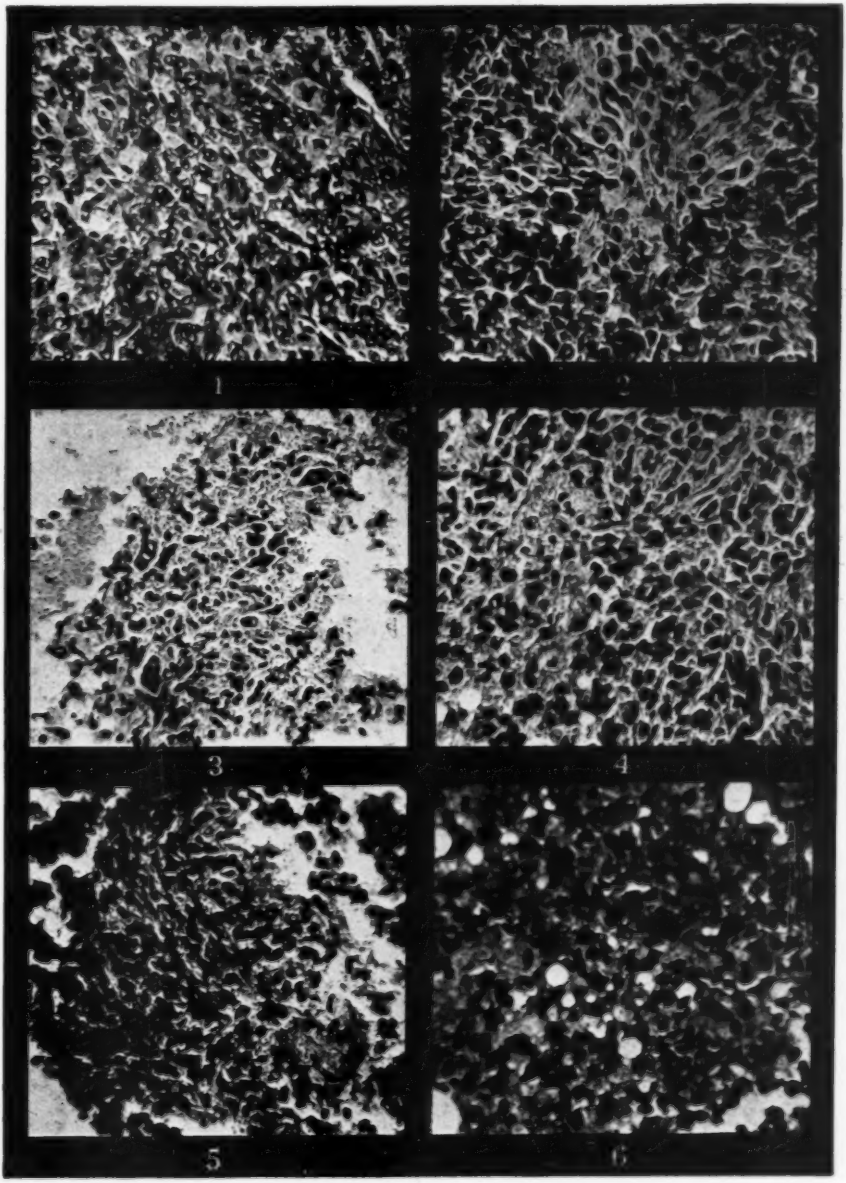
left untreated or exposed to low temperatures was proportional to the tumour-producing activity of the particular preparation being studied (Fig. 1). This is what would be expected if cell survival is assumed to be responsible for tumour production in these cases.

On the other hand, it would be expected that repeatedly frozen and thawed and freeze-dried Sarcoma 37 mince, which in our hands have never produced tumours (Warner and Gostling, 1950), would not contain " T " cells. In fact, there were very few cells, if any, which remotely resembled " T " cells, and these were not seen after the first day.

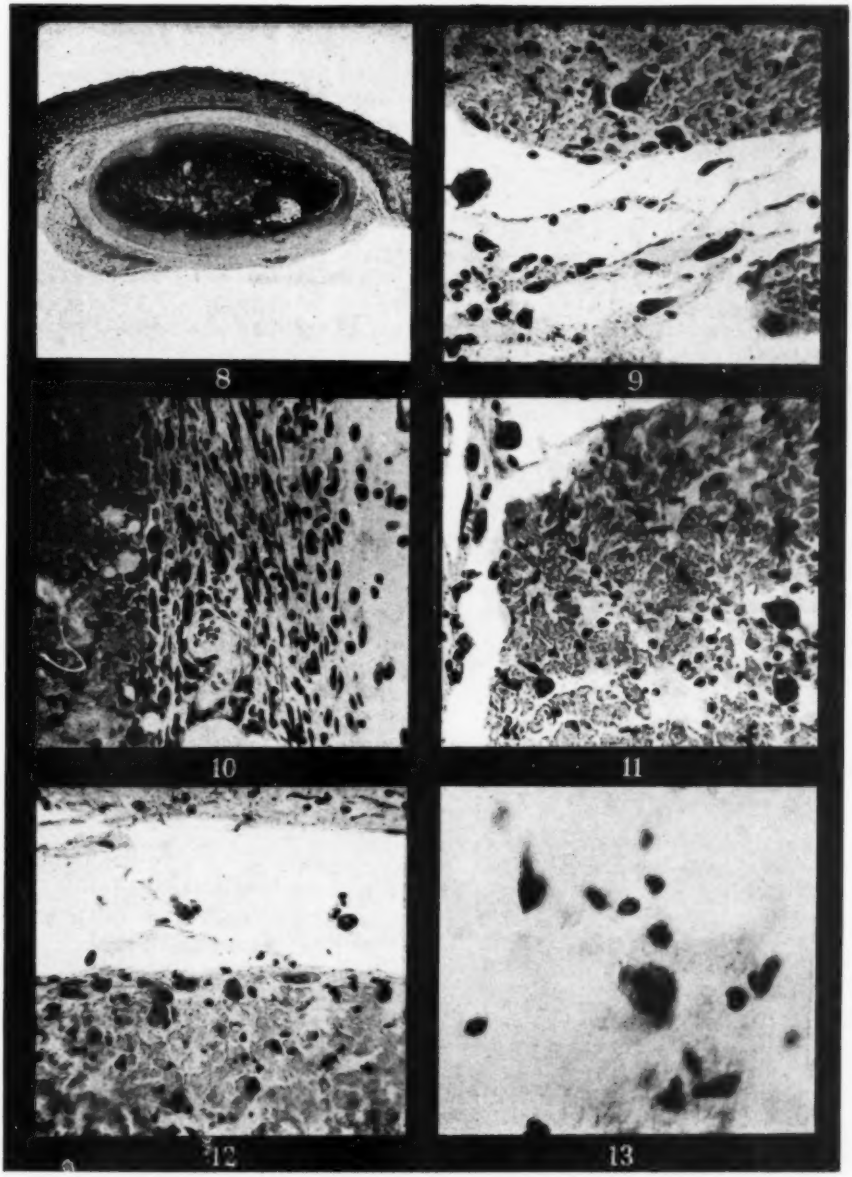
Mitoses were not seen in any preparation in undeniably characteristic " T " cells. However, they were observed occasionally in fibroblasts in the connective tissue surrounding the grafts. They were also seen within grafts of mince which had been repeatedly frozen and thawed and freeze-dried in cells which undoubtedly were fibroblasts. Two (one is shown in Fig. 13) were seen in a graft of repeatedly frozen and thawed material removed on the third day. These cells had some slight resemblance to " T " cells (Fig. 13). In short, mitotic figures were seen in cells in grafts of tissue which were known not to produce tumours, and some such cells were certainly fibroblasts and two could possibly have been " T " cells. Now, if these cells in mitosis were all fibroblasts then the presence of mitotic figures does not necessarily signify that cells, introduced with the graft, are still surviving in it. It is more likely that such cells were the forerunners of fibroblastic organization. If some of these cells in mitosis were tumour cells introduced with the graft, then the only reasonable explanation of their presence in mince which never produces tumours, is that repeated freezing and thawing or freeze-drying damages the motility of such cells and that they were attempting to divide within the necrotic graft ; whereas, had they possessed normal motility, they would first have migrated to favourable surroundings in nearby host connective tissue before attempting to multiply. Such an explanation could account for the conflicting findings of Warner and Gostling (1950), who were unable to produce tumours from freeze-dried Sarcoma 37, and Passey and Dmochowski (1950) who were able to do so. The last two workers, by reason of using glucose

EXPLANATION OF PLATES.

FIG. 1.—Sarcoma 37. × 300.
FIG. 2.—Preparation of Sarcoma 37 tissue minced with the Craigie pressure mincer. × 300.
FIG 3.—Preparation of Sarcoma 37 mince frozen slowly to — 75° C. × 300.
FIG. 4.—Preparation of Sarcoma 37 mince frozen quickly to — 75° C. × 300.
FIG. 5.—Preparation of Sarcoma 37 mince repeatedly frozen and thawed six times. × 300.
FIG. 6.—Preparation of Sarcoma 37 mince freeze-dried and reconstituted. × 300.
FIG. 8.—Low-power view of a representative graft (slowly frozen, removed after 4 days) to show fibrous capsule, peripheral rim of necrotic graft, and central area containing polymorphs and cell débris. × 9.
FIG. 9.—Showing " T " cells at edge of graft of untreated material removed after 2 days. Also showing a similar type of cell in the gap between graft and host tissues. × 300.
FIG. 10.—Showing collections of " T " cells in connective tissue near capillary at edge of a graft of untreated material removed after 3 days. × 300.
FIG. 11.—Showing two " T " cells in the peripheral rim of a graft of slowly frozen material removed on the second day. A similar cell is seen adjacent to the connective tissue surrounding the graft. × 300.
FIG. 12.—Showing " T " cells in the peripheral rim of a graft of slowly frozen material removed on the second day. Two similar cells are seen in the gap between graft and host connective tissue. × 300.
FIG. 13.—High-power view of a cell in motisis within a graft of repeatedly frozen and thawed material removed on the third day. × 1100.



Warner, Gostling and Thackray.



Warner, Gostling and Thackray

when drying, or using a different strain of tumour, or a different strain of mice, or of some other circumstance, may have provided better conditions for the few surviving tumour cells enabling them to produce tumours in 5 cases out of 10. However, the present authors think it more probable that all cells seen in mitosis in the graft were fibroblasts, because of their appearance and also because they were found after 2 days, when fibroblastic activity was well marked and increasing in amount, whereas " T " cells had a slightly different morphology, were most prominent on the first and second days, and subsequently disappeared.

The conclusions are : that tumour growth from grafts under the conditions described here depends upon the survival of a few cells which migrate into and grow in the surrounding connective tissue ; that such cells are damaged by freezing, more so if the freezing is quick than if it is slow ; that the cells are either destroyed by repeated freezing and thawing or freeze-drying, or are so damaged that they cannot migrate to a region where they can initiate tumour growth. Finally, it is concluded that the presence of mitoses within a graft is not necessarily evidence that cells, introduced with the graft, have survived.

SUMMARY.

Grafts of Sarcoma 37 mince, which had previously received no treatment or had been frozen, slowly or quickly, or repeatedly frozen and thawed six times or freeze-dried were made in mice. The grafts were removed at various intervals after inoculation varying from 3 hours to 10 days, and were examined histologically.

Sections of all grafts showed the following features : Necrosis of all, or nearly all, cells occurred early, followed closely by polymorph infiltration, which reached a peak and then died away leaving a peripheral rim of necrotic grafted tissue ; fibroblast and capillary proliferation in the surrounding tissues occurred later, formed a capsule around the graft and finally invaded its substance.

In the peripheral rim of grafts of untreated material, which were removed at about 24 hours, characteristic large cells appeared, irregular in outline, and with large round or irregular nuclei. Later, similar cells could be seen in the gap between graft and host tissues and then in the tissues themselves where collections of such cells near capillaries could be distinguished. At this time the number of such cells in the peripheral rim of the tumour graft diminished. These cells resembled those of early tumour growth which did not occur in the graft itself but in the surrounding host connective tissues. These characteristic cells, thought to be tumour cells, were designated " T " cells.

In grafts of untreated, slowly or quickly frozen mince the number of " T " cells was roughly proportional to the tumour-producing activity of the material in question.

In grafts of repeatedly frozen and thawed or freeze-dried material, which were known not to produce tumours, there were no typical " T " cells. However, an occasional cell in mitosis was seen in these preparations whereas there were none seen in untreated or frozen material.

It was concluded : that in grafts of Sarcoma 37 a few cells survived, migrated to the surrounding connective tissue of the host, and there produced tumours ; that transmission of tumours with frozen material depends upon the survival of such cells ; that these cells are either destroyed by repeated freezing and

thawing or freeze-drying or are so damaged that they cannot migrate to a region where they can initiate tumour growth. Finally, it is concluded that the presence of mitoses within a graft is not necessarily evidence that cells introduced with the graft have survived.

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PRIMARY LIVER TUMOURS IN RATS FOLLOWING FEEDING WITH ALKALOIDS OF *SENECIO JACOBAEA*.

J. W. COOK, E. DUFFY AND R. SCHOENTAL.

From the Chemistry Department, The University, Glasgow, W.2, and the Glasgow Royal Cancer Hospital, Glasgow, C.3.

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THE high incidence of primary liver cancer among the South African Negroes and the absence of any similar predominance of this rare type of cancer in the negro population of the United States suggest that some extrinsic factor is involved (Kennaway, 1944). Our colleague Mr. F. Schwarz drew our attention to a published statement that plants of the common *Senecio* species are being used indiscriminately by the South African negroes for treatment of a variety of diseases. Instances of the use of various *Senecio* plants (of which about 150 species are known to occur in Africa) by different South African tribes are recorded by Watt and Breyer-Brandwijk (1932); from the information given it would appear that starting from early childhood the negroes consume the alkaloids of these plants intermittently throughout the whole life.

Thus Sutos administer *S. asperulus* D.C. to charm away nightmares in children; Quenas and Chunas administer a milk concoction of the root of another *Senecio* plant to infants as a stimulant; Sutos smoke the leaf of *S. phyncholaenus* D.C., or inhale the smoke from burning it to treat colds; they drink a concoction of the root for colic, and mix the leaf of *S. crubescens* D.C. with their tobacco. Zulus use different *Senecio* plant infusions as blood purifiers in syphilis, chest pains, swollen gums, etc.

Senecio plants have been shown to be responsible for liver damage in cattle and horses observed in chronic poisoning known under a variety of names, as Molteno sickness, Dunsiekte, as Winton disease in New Zealand, as Pictou disease in Nova Scotia, etc. (Cushny, 1911; Cushny and Watt, 1920).

Experimentally similar conditions have been reproduced by means of the extracted alkaloids in cattle, mice, rats, hamsters and monkeys (Davidson, 1935; Rosenfeld and Beath, 1945). The experimental animals exhibited hepatic necrosis with much congestion and haemorrhage suggestive of cavernous haemangioma; they showed fibroblastic proliferation and regeneration of bile ducts, etc. In all the recorded experiments the survival of the animals never exceeded three months, but the liver changes suggested that these might have developed into neoplasia if the animals had been allowed to live longer.

The following experiments have been undertaken with the view to allowing the animals to survive for long periods.

MATERIAL AND METHODS.

Albino rats bred locally from a commercial stock and fed with rat cake (supplied by North-Eastern Agricultural Co-operative Society, Ltd., Aberdeen) and

water *ad libitum* received intermittently in their drinking water alkaloids extracted from *Senecio jacobaea* L., the common ragwort (supplied by a local herbalist). Barger and Blackie (1937) recorded the presence of three alkaloids in *S. jacobaea* plants, namely, jacobine, jacodine and jaconine, but stressed the fact that the yield and composition of alkaloids depend on the season in which the plants have been collected (e.g. plants collected in August contained only jacobine, the principal alkaloid). The season in which the plants supplied have been collected is not known to us ; the once crystallised alkaloids used in our experiments were almost certainly a mixture. We noted also that two batches of plants differed in their alkaloid content, and in the toxicity of the latter to animals.

Alkaloids of *Senecio jacobaea* belong to the pyrrolizidine group of alkaloids. On alkaline hydrolysis they yield the amino-alcohol retronecine and acids termed "necic acids." The structure of retronecine has been established by Adams and Leonard (1944). The constitutions of the acidic components have not been established (Henry, 1949 ; Leonard, 1950). The hepatotoxic effects have been produced only by the alkaloids, and have not been observed after administration of the individual products of hydrolysis, the necins or necic acids ; the animals either died immediately or recovered rapidly and completely (Harris, Anderson and Chen, 1942; Rose, Fink, Harris and Chen, 1945).

Three experiments were performed on 17 young rats weighing 31 to 88 g. at the beginning of treatment, and their weights were recorded at approximately weekly intervals till death. The consumption of the alkaloids greatly retarded growth of the animals ; some of the treated rats did not reach a weight of 100 g. till after about 6 to 10 weeks (the controls reached that weight in about 2 to 3 weeks). *Senecio* alkaloids were dissolved in the smallest quantity of dilute acetic or hydrochloric acid and the solutions diluted to contain 0·1 mg./ml., and given instead of drinking water to the experimental animals *ad libitum*. The mortality was high, especially in the early weeks of treatment ; the solutions were therefore withdrawn for a time and replaced by tap-water. This procedure has been repeated intermittently ; withdrawal took place whenever one of the animals died, or when the general health appeared unsatisfactory. Eventually the concentration of the solution was reduced to 0·05 mg./ml.

Autopsies were performed on each animal which died and on those which were sacrificed at intervals ; livers and other organs were histologically examined, except those of animals which died in the first two weeks of treatment.

RESULTS.

Animals are grouped according to the similarity of the histological appearances of the liver. These are :

(*a*) Necrosis and degenerative change in persisting parenchyma.

(*b*) Degenerative change in persisting cells and evidence of diffuse regeneration, and hyperplasia of bile ducts.

(*c*) Nodular regeneration, hepatoma and bile duct cystadenoma.

Cirrhotic change is not seen in the present material, and inflammatory infiltration is absent or minimal around the portal tracts. A summary of the results is given in Table I. The number of animals and the duration of experiments are given in the subheadings.

TABLE I.—*Summary of Changes Observed in Liver.*

Number.	Duration of experiment in months.	Abnormal macroscopic characters.	Histology.
516	1	Pale yellow colour	Necrosis and degenerative change
498	1	,, ,,	Ditto
499	1	,, ,,	,,
515	1	Slight diminution in size; pale yellow colour	,,
567	2	Yellow colour	Degeneration and hyperplasia
497	6	Slight increase in size; golden-brown colour	Ditto
511	6½	Yellow colour	,,
569	7	Green colour	,,
423	8	Nodularity on surface of left lobe with solid nodules of pale colour in substance	Degeneration, diffuse and focal regeneration
568	10	Increase in size via exaggerated lobulation and presence of ovoid tumour in left lobe	Degeneration, hyperplastic change, hepatoma, cholangioma
454	11	Slight increase in size and exaggerated lobulation	Degeneration, nodular regeneration, hepatoma.

Group (A): Four animals treated 1 month.

Animals treated for about 1 month show pale yellow livers of normal shape and smooth surface. One liver appears slightly diminished in size; the others are within normal limits. The histological characters are those of acute necrosis occurring irregularly, and sometimes extending from the periportal region to the mid-zone of the lobule. Persisting liver cells often arranged in close apposition to one another around the central lobular vein exhibit an appreciable increase in size, poorly defined cell line and considerable irregularity in shape. Some appear to fuse, forming syncytia, and many others show double nuclei. The cytoplasm is well packed with finely granular material, which is acidophil in the periphery and basophil in the perinuclear zone. Intra-cytoplasmic vacuolation is sometimes seen.

The nuclei are very variable in size and show an inconstant chromatin pattern which, in some, is quite dense, and in others, open and vesicular, with usually more than one acidophil nucleolus. All stages of transition between viable cells and necrotic forms are seen at the edge of the persisting columns, the signs of cell death being lysis of nucleus and uniform dense acidophil character in the cytoplasm. Where liver cells are absent without trace the space is occupied by an open framework of fine reticular tissue.

There is no dilatation of the sinusoids, and the bile ducts persist without evidence of proliferation.

Group (B): Five animals treated 2 to 8 months.

The livers show abnormal coloration from yellow to golden brown; one is bright green. In respect of size, shape and smooth surface, all are normal except one, in which the left lobe has an irregular surface and small grey nodules about 1 mm. in diameter in its substance. Microscopically they are very cellular and show no distinct lobular pattern. The liver substance is composed alternately and in varying proportions of enlarged degenerate cells showing the characters seen in Group (A), and smaller regenerating forms diffusely distributed or crowded together without definite arrangement. The latter are fairly regular in size and shape, and sometimes occur as small pseudo-encapsulated nodules. Many show clear cytoplasmic vacuoles. In some cells these cause compression of the nucleus, which may appear as a dented spheroid, or as a crescent. Small foci of atrophy are sometimes seen. The bile ducts are prominent, and in some places show marked hyperplastic change.

Group (C): Two animals treated 10 to 11 months.

The livers of both animals show slight or definite increase in size with exaggerated lobulation. In the left lateral lobe of one an ovoid light brown tumour mass (diameter 1·5 × 1 cm.) is present; in the other a whole lobe is transformed into a tumour. In each case the liver is very cellular, and shows characters similar to those described in Group (B). Hyperplastic liver tissue, however, is more prominent in this group, and the cells are seen diffusely distributed throughout the original hypertrophic parenchyma and as solid, structureless, but sometimes trabeculated nodules compressing the surrounding parenchyma.

In addition to these features, there occur foci of proliferation where frankly neoplastic characters are seen. There is variation, though limited, in cell form and character, and a moderate degree of nuclear aberration. Mitotic figures are seen, but are not numerous. The cells are arranged in various patterns, including the solid alveolar type, and the open sinusoidal arrangement with blood in the interspaces. In the latter small imperfect acini are sometimes seen. There is proliferation of the bile ducts, and in one field a cystadenoma of bile duct epithelium is present.

Control animals.

No abnormal features seen in the liver.

Changes in other organs.

Subcutaneous oedema with pleural or peritoneal effusion of clear serous fluid was noted in animals dying before 6 months of treatment. These and other animals showed haemorrhage into the gastro-intestinal tract. A moderate degree of fibrosis affecting the pulp of the spleen was seen in two animals, and degenerative change affecting the epithelium of the convoluted tubules of the kidney in one.

DISCUSSION.

Senecio alkaloids administered orally are directly toxic to liver cells, and produce either coagulation necrosis or degenerative change. Subsequently regeneration takes place, under favourable circumstances, and may be followed by

nodular hyperplasia of liver cells, or the formation of tumour-like masses showing the characters of hepatomas and excessive proliferation of bile duct epithelium. The lesions are essentially similar to some of those produced by *p*-dimethylaminoazobenzene (butter yellow), and described by many workers, notably by Opie (1944).

In the absence of metastases it is not certain that the changes observed represent stages of an irreversible neoplastic process. There is little reason to doubt, however, that with survival over a longer period truly malignant characters may supervene.

The finding that liver tumours are produced in rats treated intermittently for more than 8 months with alkaloids of *Senecio jacobaea* raises interesting problems. The question how far the results described above bear on the problem of primary liver tumours in man suggests clinical inquiry about herbal treatment of the respective patients. However, the prevalence of this type of tumour among populations known to use concoctions of *Senecio* plants from childhood suggests that this practice may play a part in the aetiology of primary liver tumours.

Senecio plants are being still sold in Great Britain for treatment of colds, coughs, sciatica, pains in limbs and other disorders (Potter and Wren, 1941; Gerarde, 1597). The recommended dose of an infusion of one ounce of dried plant in a pint of water is a wineglassful. Assuming the alkaloid content of the dried plant to be of the order 0·06 per cent (Barger and Blackie, 1937), such an infusion would contain about 0·03 mg./ml., a concentration comparable with that used in our experiments (0·05–0·1 mg./ml.). In view of the chronic or recurrent nature of the disorders for which such treatment is recommended, its danger is obvious.

Recently, Hoch-Ligeti (1949) reported the production of liver tumours in 12 out of 21 rats killed after 7 months' feeding of 10 per cent. chilli (*Capsicum fruitescens* and *C. annuum*) in a semi-synthetic deficient diet; this contained ardein, the protein of ground nuts. In a previous experiment of the same worker (1948), when the rats received 10 per cent chilli in a semi-synthetic diet, containing, however, an animal protein, casein, for 14 to 24 months, 7 out of 50 experimental rats developed liver changes resembling hepatoma and cholangioma, three of them malignant. In these experiments the type of diet seems to have had a contributory action. In our experiments the animals were fed the well-balanced rat cakes *ad libitum*. However, the weight of the rats was markedly influenced by the consumption of the alkaloids, and was below that of the controls.

That deficient diet of the type used by the Bantus is not sufficient to induce liver tumours has been shown by the experiments of Gillman (1944), and Gillman, Gillman, Mandelstam and Gilbert (1945). These workers fed newly weaned albino rats with maize-meal porridge (mealie-pap) and sour milk (the staple diet of the South African negro) for up to 15 months. Although severe liver injury was produced, no tumours developed.

SUMMARY.

Liver tumours have been observed in 3 albino rats which survived more than 8 months of intermittent feeding with alkaloids of *Senecio jacobaea*. The possible bearing of these results on the aetiology of primary liver tumours frequent among the negro population of South Africa is discussed in the light of the indiscriminate use of *Senecio* plants for treatment of numerous disorders.

These experiments were begun in the animal house of the Materia Medica Department, where facilities were kindly placed at our disposal. We are greatly indebted to Professor C. M. Yonge, F.R.S., for the provision of alternative accommodation in the Zoology Department, which enabled the work to be continued when these facilities were withdrawn. Our thanks are due to Miss E. M. Ross and Miss E. B. Duff for excellent care of the animals, and the former for valuable technical assistance ; also to Mr. S. Breslin for photographic reproductions and histological sections. This work has been supported by a grant from the British Empire Cancer Campaign.

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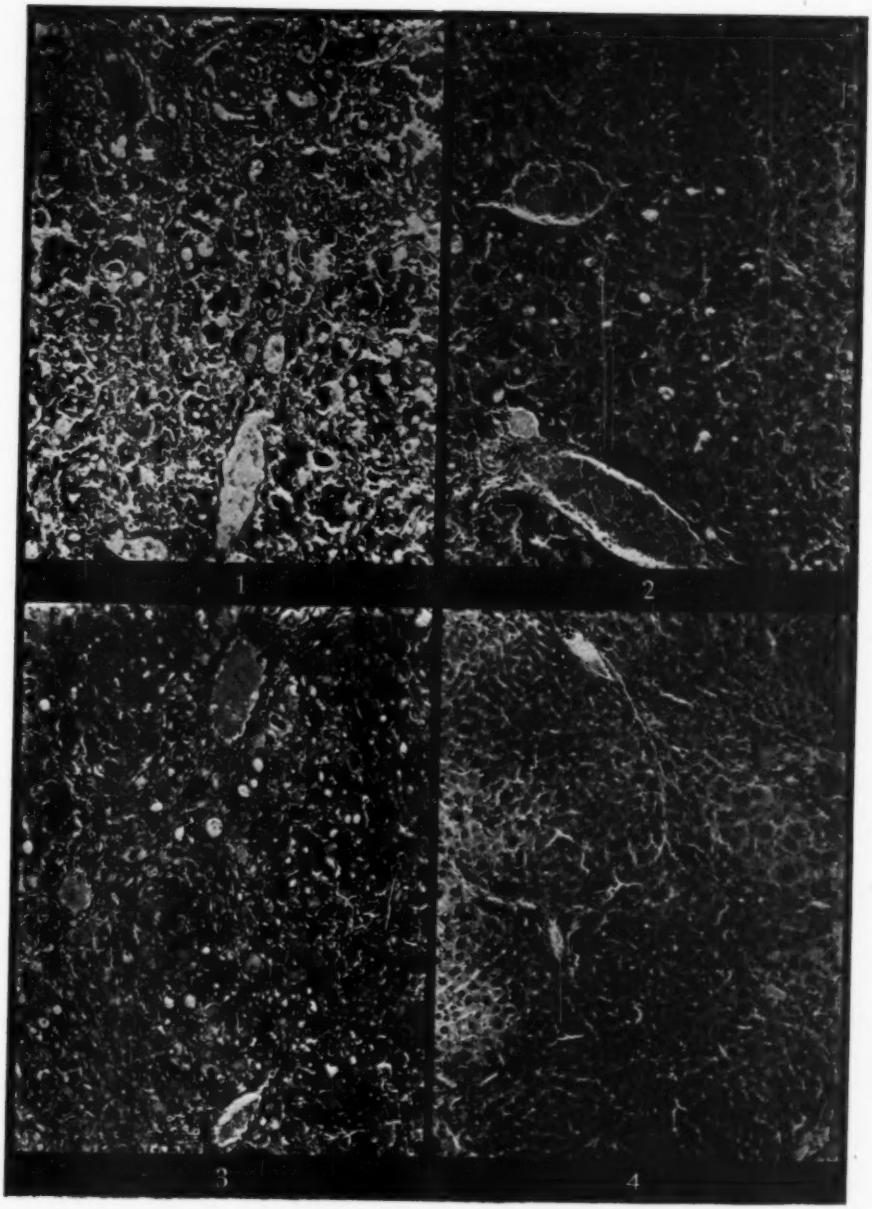
EXPLANATION OF PLATES.

FIG. 1.—Section 516 of liver from a rat which died during the first month of treatment with *S. jacobaea* alkaloids, showing necrotic and degenerative changes.
FIG. 2.—Section 511 of liver of a rat which died after 6½ months of treatment, showing besides degenerative-hyperplastic changes ; note cytoplasmic vacuolation, abnormal and variable nuclear patterns.
FIG. 3.—Section 569 of liver from a rat which died after 7 months' treatment showing features similar to Fig. 2.
FIG. 4.—Section 423 of liver from a rat sacrificed after 8 months of treatment showing hepatomatous growth (diffuse and focal regeneration).
FIG. 5, 6, 7.—Different areas of section 568 of liver tumour from a rat which died after 10 months' treatment.
FIG. 8.—Section 454 of liver tumour from a rat sacrificed after 11 months of treatment.



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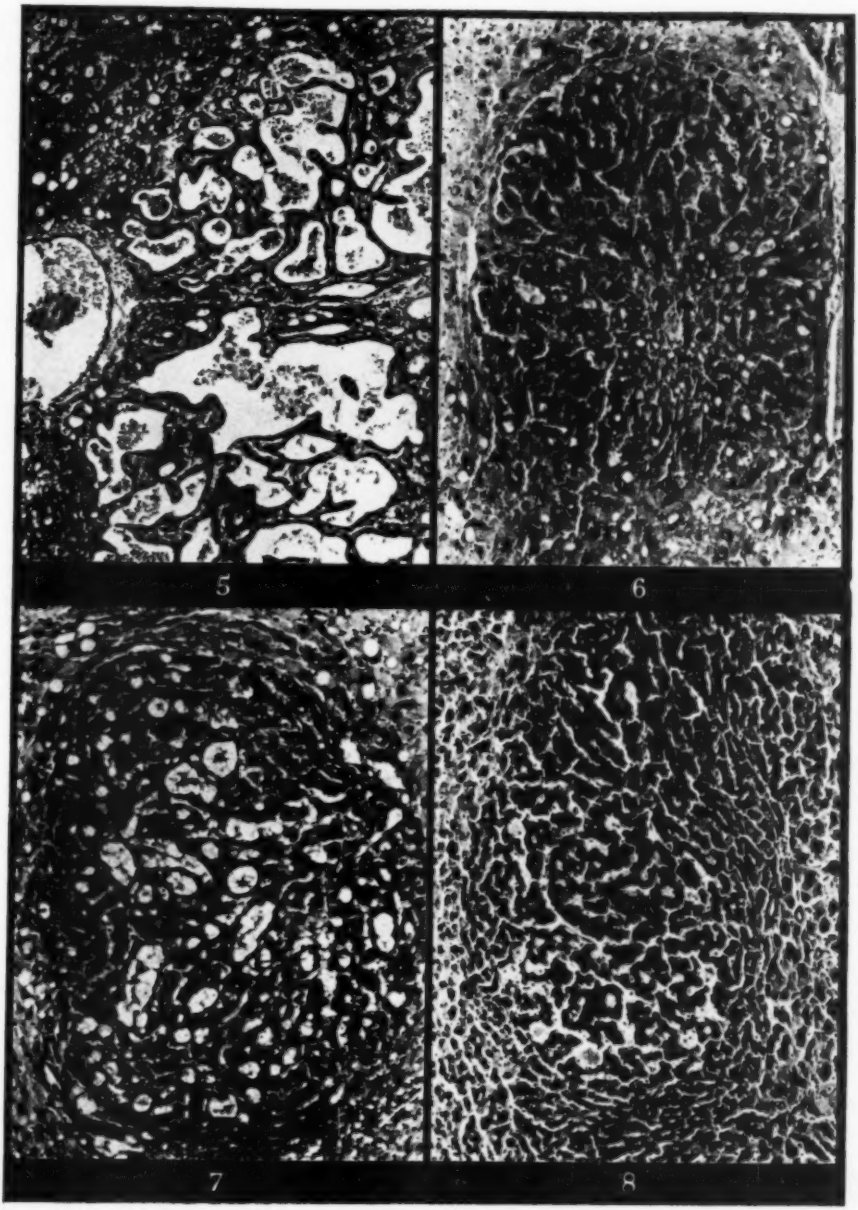

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THE CARCINOGENIC ACTIVITY OF TANNIC ACID. LIVER TUMOURS INDUCED IN RATS BY PROLONGED SUBCUTANEOUS ADMINISTRATION OF TANNIC ACID SOLUTIONS.

B. KORPÁSSY AND M. MOSONYI.

From the Department of Morbid Anatomy of the University of Szeged, Hungary.

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KORPÁSSY AND KOVÁCS (1949) recently published the first account of the successful production of liver cirrhosis in white rats by the repeated parenteral administration of tannic acid solutions in sub-lethal doses at various intervals over a long period. The macroscopical and microscopical changes in the liver at an advanced stage of experimental tannic acid cirrhosis of white rats were not found to differ in any way from those seen in human cirrhosis of the Laennec type. These investigations also indicated that the changes in the livers of experimental animals produced by regular tannic acid administration are progressive and do not come to an end with the characteristic distortion of the liver architecture, for in the livers of some of the rats which survived the 100th day of treatment there appeared a few nodules of greyish white colour and 2 to 5 mm. diameter which proved microscopically to be hepatomas or cholangeiomas. One must therefore suppose that the tannic acid may also have some tumour-producing effect. A preliminary publication of these investigations has appeared (Korpássy and Mosonyi, 1950).

EXPERIMENTAL.

The present series of experiments was planned to demonstrate both the local and remote effects of tannic acid.

A. *Parenteral Administration.*

Tannic acid solution was injected subcutaneously into 28 two months old white rats over a long period. Of the 28 animals, 14 were male, and 14 female, and their average weight at the beginning of the experiment was 68 g. Their diet consisted of mixed waste food from the hospitals; the precise fat, carbohydrate, protein and vitamin contents of the food were disregarded, as a considerable number of untreated rats of the same strain fed in the same way grew and reproduced normally. The rats were of the same strain as those used in the earlier experiments (Korpássy and Kovacs, 1949).

At first 150 mg., later 200 mg. of tannic acid per kg. body weight as a 1·5 to 2 per cent aqueous solution was administered subcutaneously, usually every 5th day, on the backs of all the animals. The tannic acid used was Acid. tannic U.S.P. obtained through Johnson & Sons, Ltd., Hendon, London, N.W.4. The weights of the animals were systematically controlled, the weight curve usually

showing a steady rise until the 90th day of treatment, when an average weight of 130 g. was reached. If any animal showed a considerable loss of weight, treatment was discontinued for some days to avoid its death early in the experiment. Apart from slight fluctuations the body weights continued to increase, reaching an average of 178 g. on the 150th day. Up to the 300th day of treatment and over, respectively, the average weights of the animals were 150 and 200 g.

In the first third of the treatment period 6 animals died. By the 100th day of treatment 23 animals still survived, 12 survived 200 days, and only 5 animals survived 300 days. Treatment was discontinued on the 290th day ; rats surviving this period had received altogether 49 subcutaneous injections of tannic acid solution. The weights of the animals did not exhibit any marked change after stopping the treatment. One rat was killed on the 358th, another on the 363rd, and the last one on the 388th day.

B. *Skin Painting.*

In order to study both local and remote effects of the skin ulcers a patch of skin the size of a two shilling piece on the backs of each of 39 white rats was burned with a glowing spatula. When the scabs had peeled off we tried to check the healing of the ulcers. The ulcers of 20 rats were painted daily with 5 per cent fresh aqueous tannic acid solution, while the ulcers of 19 rats were similarly painted with 5 per cent hydrochloric acid. If healing of the ulcers progressed in spite of this treatment, the skin was burned again or treated with concentrated hydrochloric acid. This treatment had to be repeated at intervals of 6 weeks. Of the group treated with tannic acid 14 rats survived 300 days, and 11 rats 400 days ; of this group treated with hydrochloric acid 10 rats survived 300 days, and 9 animals 400 days. Two animals from each group were killed on the 505th day. The animals dying during treatment were found to have succumbed to various diseases, such as lung abscess, enterocolitis and otitis media.

In this experiment six months old white rats were used, their average weights at the beginning being 161 g. for the tannic acid group and 171 g. for the hydrochloric acid group. The animals were of the same strain and received the same diet as in Experiment A.

For histological examination the tissues were fixed in 4 per cent formaldehyde, embedded in paraffin wax and sections were stained with haematoxylin and eosin, van Gieson's stain, and Gömöri's stain for reticulum. The livers were examined in all cases ; other organs only in the few instances in which a macroscopic lesion was present.

CHANGES PRODUCED.

Local effects.

The repeated subcutaneous administration of 1·5 to 2 per cent aqueous tannic acid solution resulted in necrosis of the skin at the site of injection, and ulcers remained after separation of the necrotic tissue (Experiment A). Although fairly large skin ulcers were produced in animals treated for long periods, they healed rapidly and completely on changing the site of injection or temporarily discontinuing it, and did not seem to influence the general health of the animals. In no case did a tumour arise from the margin of an ulcer or from the healed scars.

The local treatment with tannic acid and with hydrochloric acid of the skin ulcers produced by burning (Experiment B) yielded no result at all. The skin, i.e. the skin ulcers, of 11 rats was painted daily with 5 per cent tannic acid solution over a period of 400 days (2 animals were killed on the 505th day of treatment), but no change of any kind appeared at the site of application in any animal; on the contrary the tendency to heal of the ulcers produced by burning in rats painted with tannic acid seemed somewhat more pronounced than that of those treated with hydrochloric acid.

Remote effects.

The livers of several of the 23 rats surviving the 100th day of subcutaneous tannic acid treatment (Experiment A) showed different degrees of early or advanced diffuse nodular cirrhosis, and associated with such changes or without them there arose hepatic tumours which varied in size and structure.

On the other hand, in the livers of the animals with skin ulcers painted with tannic acid or hydrochloric acid solution for a long while, no change which could be connected with the treatment was observed, even in those treated for the longest period. In none of the animals were cirrhotic or precirrhotic changes observed, nor was there any increase of the reticulum fibres in the liver. Similarly, in no case did a hepatoma or cholangeioma occur in the livers of these rats, though they were 4 months older at the beginning of the experiment and most of them survived 100 to 200 days longer than those treated with subcutaneous tannic acid solution.

Gross pathology.

The findings referred to below concern only those animals which had the subcutaneous tannic acid solution treatment (Experiment A).

No noteworthy changes were observed in the liver during the first 2 months except for blurring or occasionally exaggerration of the lobular pattern. The first definite naked eye changes were seen in a rat which died on the 109th day, and in whose liver there were a few nodules of greyish-white colour and the size of a pinhead or millet seed located beneath the capsule. It was thought that these represented the earliest tumours, and histological examination seemed to confirm this.

On the 121st and 122nd days 6 rats died and in the liver of one of them there were well marked and advanced changes. The surface of the liver was rendered granular by the presence of a large number of nodules beneath the capsule, the size of millets or peas and of a greyish-white colour, the intervening liver tissue being reddish-brown. In addition to these nodules, in one of the lobes there was a fairly solid tumour $5 \times 6 \times 4$ mm., sharply defined and also greyish-white in colour (Fig. 1). This picture differs completely from the diffuse nodular cirrhosis produced by the tannic acid (Fig. 3), and macroscopic examination left no doubt as to the neoplastic nature of the nodules. The cut surfaces of the liver of a rat which died on the 231st day showed a very marked miniature nutmeg pattern with several greyish-white nodules, the size of poppy seeds or millets (Fig. 2); though such nodules are recognizable in unfixed livers, after fixation they are more obvious. The liver of the rat that died on the 287th day showed the most marked diffuse nodular cirrhosis. The surface of all the lobes was

evenly granular, the brownish-red granules being sharply defined, the size of millets or peas, and with darker coloured fine furrows among them (Fig. 3). On section the nodular appearance was still obvious, with loss of the normal pattern ; and, in addition, on the cut surfaces there were visible some nodules, the size of beans, of a much clearer greyish-white colour than the adjacent cirrhotic liver tissue and which were definitely tumour-like.

After 290 days when the administration of tannic acid solution was discontinued, the macroscopic appearance of the livers—apart from the tumours —gradually reverted to normal. After this time 8 animals died or were killed, and the majority of their livers appeared normal. Nevertheless, 6 of these livers showed solid tumours which were no larger than those described above, and were usually well circumscribed and paler than the surrounding liver tissue. Some of the larger ones had a moderately lobulated appearance.

Ascites appeared in some of the animals, especially those in which the cirrhosis seemed to be most advanced, but varices of the portal-systemic venous anastomoses were never detected. No metastases were seen.

Microscopic pathology.

In the livers of the rats that died during the first two months of the parenteral tannic acid treatment there was extensive necrosis involving the central one-third to one-half of the lobules. Necrosis of sinusoidal endothelium was not apparent. There were numerous mitotic figures in the viable peripheral portions of the lobules and regeneration was evident throughout the greater part of tannic acid administration. While many of the mitoses appeared normal, atypical forms were not infrequent. The central necrotic cells were progressively phagocytosed, and for the greater part removed. The swelling and multiplication of the reticuloendothelial cells, mainly in the centre of the lobules, were seen quite early, while the reticulum fibres in the same region became thick and numerous. Bile duct proliferation was most well marked about the end of the second month.

In the animals surviving the 100th day of tannic acid administration the architecture of the liver was greatly disturbed. There was an appreciable increase in reticulum and sometimes in connective tissue, distributed mainly in the vicinity of the portal tracts, but also extending away from these areas in an irregular fashion and cutting up the parenchyma into lobules of irregular size and shape.

The Tumours.

The induced tumours of the liver, although showing a variety of pictures, could be divided histologically into two main groups, hepatomas and cholangeiomas.

Hepatomas.—Of the hepatomas two types can be distinguished, a well differentiated and a less differentiated one, the former as a rule appearing as smaller nodules. The well differentiated type was sometimes encapsulated, compressing the adjacent hepatic tissue which often showed a local increase in reticulum (Fig. 4). The characteristic feature of the hepatomas was that the epithelial cells were arranged in cords which were separated from the endothelial lined sinuses by delicate slips of reticulum. In the well differentiated form the tumour cells had prominent cell margins, a granular, occasionally vacuolated, acidophilic cytoplasm and relatively large vesicular nuclei with one to three prominent,

often acidophil nucleoli (Fig. 5). So closely did this form resemble hepatic tissue that it was sometimes an open question whether a given lesion should be regarded as an example of active non-architectural regeneration or as neoplastic. In such cases the former interpretation was adopted for statistical purposes.

In the larger hepatomas the cord-like arrangement of the tumour cells was lost or there were cords of variable thickness. Acinar structures were not infrequently encountered made up of cells closely resembling hepatic parenchymal cells (Fig. 6). The hepatoma cells showed considerable variation in size, their margins were somewhat obscure and the cytoplasm was usually faintly basophilic ; in most of the tumours the cells were larger than normal hepatic cells. Mitotic figures varied considerably in number and some were atypical (Fig. 7). No centrally placed blood vessel or bile ducts were found in the hepatomas. In some of the larger hepatomas slight fatty degeneration and areas of focal necrosis were seen. The connective tissue of the tumours was usually scanty. In some tumours there was invasion of hepatic blood vessels (Fig. 8, 9).

Proliferation of bile ducts.—Proliferation of the bile ducts could often be detected before the 100th day, sometimes reaching an extent which might justify the description adenomatous. In rats surviving the 100th day of treatment small areas of bile duct proliferation were not infrequently found, showing an abundance of reticulum separating the ducts. Opie (1944) described this change in the livers of rats treated with *p*-dimethylaminoazobenzene and called it cholangeiofibrosis.

Besides these changes, the neoplastic nature of which is debatable, a more extensive and markedly irregular bile duct proliferation could be observed in quite a number of cases (Fig. 10). Tubules varied considerably in shape and width, and were surrounded by only a small amount of connective tissue. Still more marked irregularities may be seen in the epithelial lining ; the cuboidal or columnar epithelial cells were often markedly pleomorphic in shape and size, their nuclei being sometimes vesicular, sometimes hyperchromatic, and the cells were sometimes more than one layer thick. In addition to the increase in the nuclear-cytoplasmic ratio, the presence of solid acini, with numerous and atypical mitoses were features which made distinction from the non-neoplastic bile duct proliferations easy. Finally, the fact that not infrequently small islets of liver cells could be found incorporated in the mass of proliferating bile ducts proved the infiltrative growth of such tumours (Fig. 11, 12). Lesions of this type have been called by us cholangeioma. Just as the regenerative and neoplastic proliferations of bile ducts cannot be sharply distinguished, so there are also transitional forms among the cholangeiomas produced where the diagnosis between benign adenoma and low grade adenocarcinoma is very difficult.

Incidence of induced liver tumours and cirrhosis.

The frequency of hepatomas and cholangeiomas produced by parenteral tannic acid treatment with regard to period of treatment is represented in Table I. Taking into consideration only the 23 animals that survived the 100th day of treatment and were killed or died between days 109 and 388, we succeeded in inducing hepatic tumours in 13, or about 56 per cent. From the data in Table I it would seem that the final incidence of tumour formation might be greater,

TABLE I.—*Liver Tumours Produced by Parenteral Tannic Acid Treatment.*

Days of experiment.	Number of rats examined.	Average survival time (days).	Number of rats with liver tumours.			Total.
			Hepatoma.	Cholangeioma.	Hep. + Chol.	
11–64	5	36	–	–	–	–
109–185	11	128	1	3	1	5
215–295	7	265	2	2	–	4
320–388	5	353	2	1	1	4
Total	28	—	5	6	2	13

though not significantly so, because of the relatively small number of animals used. We would mention, however, that only macroscopic nodules whose neoplastic nature was established histologically have been taken into consideration. Some authors have gone further ; Crabtree (1949), investigating the carcinogenic action of aminoazotoluenes, regarded both the microscopic nodules of hepatoma and beginnings of cholangeiomas as tumours produced by his treatment.

Table II shows the connection between cirrhosis and tumour production. Cirrhosis appeared in the livers of 15 out of the 23 rats which survived the 100th

TABLE II.—*Relation between Liver Tumour Production and Cirrhosis.*

Days of experiment.	Number of rats examined.	Number of rats with liver cirrhosis.			Total.	Cirrhosis in tumour rats.			Total.
		I.	II.	III.		I.	II.	III.	
11–64	5	–	–	–	–	–	–	–	–
109–185	11	3	4	1	8	1	2	1	4
215–295	7	1	2	2	5	–	2	2	4
320–388	5	1	–	1	2	–	–	1	1
Total	28	5	6	4	15	1	4	4	9

day of treatment ; the cirrhosis is graded in the table as follows : Grade I—beginnings ; Grade II—early stages ; Grade III—developed and advanced processes. In 9 animals, simultaneously with cirrhosis of different degree, liver tumours were formed, while in 4 animals with liver tumours no cirrhosis could be detected. The sex of the animals does not seem to influence the incidence of induced liver tumours and cirrhosis.

Incidence of spontaneous tumours.

Spontaneous tumours are scarcely ever found in the strain of albino rat, our own breed, which has been used for years in all our experiments. Although quite a large number of untreated old rats was autopsied, in only a single case was a spontaneous liver tumour noticed. A typical cysticercus sarcoma with extensive omental and pulmonary metastases was found in the liver of one rat in Experiment B of this paper that died on the 425th day of hydrochloric acid skin painting. In no case was a spontaneous hepatoma, cholangeioma, benign or malignant tumour arising from any other organ observed.

Transplantation.

Subcutaneous and intra-hepatic transplants of tumour tissue were made by the trocar technique into rats of the same strain. From a bean-sized liver tumour of one animal subcutaneous transplantation was made into 5 white rats ;

no tumour was palpable even after 5 months. From a hazel nut sized liver tumour of another animal transplantation, into the livers of 5 white rats, and subcutaneously in 5 other white rats, was performed. Transplantation, even after 2 months, seems to fail.

DISCUSSION.

Considerable attention has been paid lately to the study of chemical substances producing hepatic tumours. The best known of these agents are the azo-dyes, and Sasaki and Yoshida (1935) were the first to succeed in producing liver cancer in rats fed *o*-amidoazotoluene. The most effective of the carcinogenic azo-dyes is butter yellow (*p*-dimethylaminoazobenzene). This dye is used to colour oils, candies, oleo-margarine and other vegetable fat substitutes for butter. The carcinogenic action of this dye on the liver was discovered by Kinoshita (1937). Cruz (1948) observed four types of lesion in the liver more or less in accordance with the time of survival of the rats : 1, acute serous hepatitis ; 2, adenomatosis, or bile duct adenomas ; 3, fibrosis or annular cirrhosis ; 4, stage of carcinoma or hepatoma. Eltsina (1945) painted the skins of mice with 1 per cent *o*-aminoazotoluene and after 9 months' treatment small hepatomas and cholangeiomas appeared in the livers. Morozenskája (1946) succeeded in transplanting the hepatoma of a mouse fed *o*-aminoazotoluene ; it was transplanted and grew under the skin of white mice for 37 generations.

The effect of carbon tetrachloride administered orally to mice resembles that of the azo-dyes. The carbon tetrachloride-induced tumours are well differentiated hepatomas and resemble the spontaneous and *o*-aminoazotoluene-induced tumours of the mouse. One tumour out of 8 in which transplantation was attempted, proved transplantable (Edwards, 1941). According to investigations made by Cameron and Karunaratne (1936) and others, carbon tetrachloride is a substance producing cirrhosis.

Another carcinogenic agent, the effect of which is exclusively remote, is 2-acetyl-aminofluorene. The carcinogenic properties of this agent were discovered by Wilson, DeEds and Cox (1941). Oral administration to rats of small quantities of acetyl-aminofluorene has been followed by the development of a wide variety of tumours in different tissues. The liver nodules were the commonest and most prominent lesion. Most tissues that gave rise to tumours were also the sites of nodular epithelial hyperplasia and no sharp distinction could be made between these nodules and the tumours formed by similar tissues (Cox, Wilson and DeEds, 1947).

According to our own investigations the effect of tannic acid on the liver resembles very much that of the substances here specified. Administered parenterally tannic acid produces serous hepatitis and acinocentral necrosis (Korpássy, 1949). It proved hepatotoxic when administered orally in appropriate dosage (Korpássy, Koltay and Horvai, 1950). By parenteral administration to rats for a longer period cirrhosis of the liver is produced (Korpássy and Kovács, 1949).

As is shown by the investigations published here tannic acid has no local tumour-producing effect. The morphology of the liver tumours produced by parenteral tannic acid administration, employed for the first time by the authors, parallels that described by Orr (1940), Opie (1944) and others in rats fed butter yellow ; by Cox, Wilson and DeEds (1947), Harris (1947) and others in rats fed

acetyl-aminofluorene ; or by Edwards (1942), and Eschenbrenner and Miller (1946) in mice-fed carbon tetrachloride. It is true we have not yet succeeded in producing metastases from the liver cancers resulting from parenteral tannic acid treatment, yet invasion of the hepatic blood vessels was observed in two animals, indicating that some of the tumours produced cannot be regarded as benign. Willis (1948) states that the invasion of hepatic veins is the prelude to metastasis to the lungs in human carcinoma of the liver. One of our animals, in whose liver an early hepatic carcinoma was definitely detected, died on the 122nd day. If this animal had lived a few weeks longer it seems probable that metastases would have formed.

The widely disputed question of the relation between cirrhosis and the formation of hepatic tumours cannot be left out of consideration. According to Sugiura and Rhoads (1942) the administration of *p*-dimethylaminoazobenzene first results in cirrhosis of the liver, followed later by the appearance of tumours. When using *o*-aminoazotoluene, however, no cirrhosis occurs as a rule. On the other hand, Maruya (1939), Miller, Miner, Rusch and Baumann (1941) and Opie (1944) are all of the opinion that cirrhosis is not necessary for experimental liver tumour formation. Data given by Harris, Krahl and Clowes (1947) also show that tumours develop readily in the liver in the absence of cirrhosis. Kline (1943) observed that the addition of *p*-aminobenzoic acid to the diet containing butter yellow greatly reduced the incidence of cirrhosis without changing the frequency of liver cancer. Eschenbrenner and Miller (1946) on the basis of quantitative histological studies stated that repeated liver necrosis and its associated chronic regenerative state are probably not necessary for the induction of tumours with carbon tetrachloride.

On the basis of our investigations made up to the present time we do not wish to take a definite attitude as to the relationship between cirrhosis and the formation of liver tumours. It was not easy to establish a sharp distinction between regenerative hyperplasia and neoplasia, and nodules which were not unquestionably neoplastic were classed as non-neoplastic, though numerous areas were suggestive of early neoplasm. It is, however, certain that no cirrhosis could be

EXPLANATION OF PLATES.

FIG. 1.—Rat 0/13. Treated with 4250 mg. per kg. body weight tannic acid, administered in 22 doses. Died on 122nd day. × 1.

FIG. 2.—Rat 0/19. Total 7700 mg. tannic acid per kg. body weight in 39 injections. Died on 231st day. × 1.

FIG. 3.—Rat 0/21. Total 9700 mg. tannic acid per kg. body weight in 48 injections. Died on 278th day. × 1.

FIG. 4.—Rat 0/27. Total 9950 mg. tannic acid per kg. body weight, in 49 injections. Killed on 358th day. Hepatoma and adjacent hepatic tissue. Haematoxylin and eosin. × 135.

FIG. 5.—Rat 0/27 (Fig. 4). Hepatoma and adjacent hepatic tissue. Liverlike cells with prominent nucleoli. Haematoxylin and eosin. × 235.

FIG. 6.—Rat 0/13 (Fig. 1). Acinar formation in hepatoma. Haematoxylin and eosin. × 200.

FIG. 7. Rat 0/27 (Fig. 4). Mitosis in hepatoma. Haematoxylin and eosin. × 250.

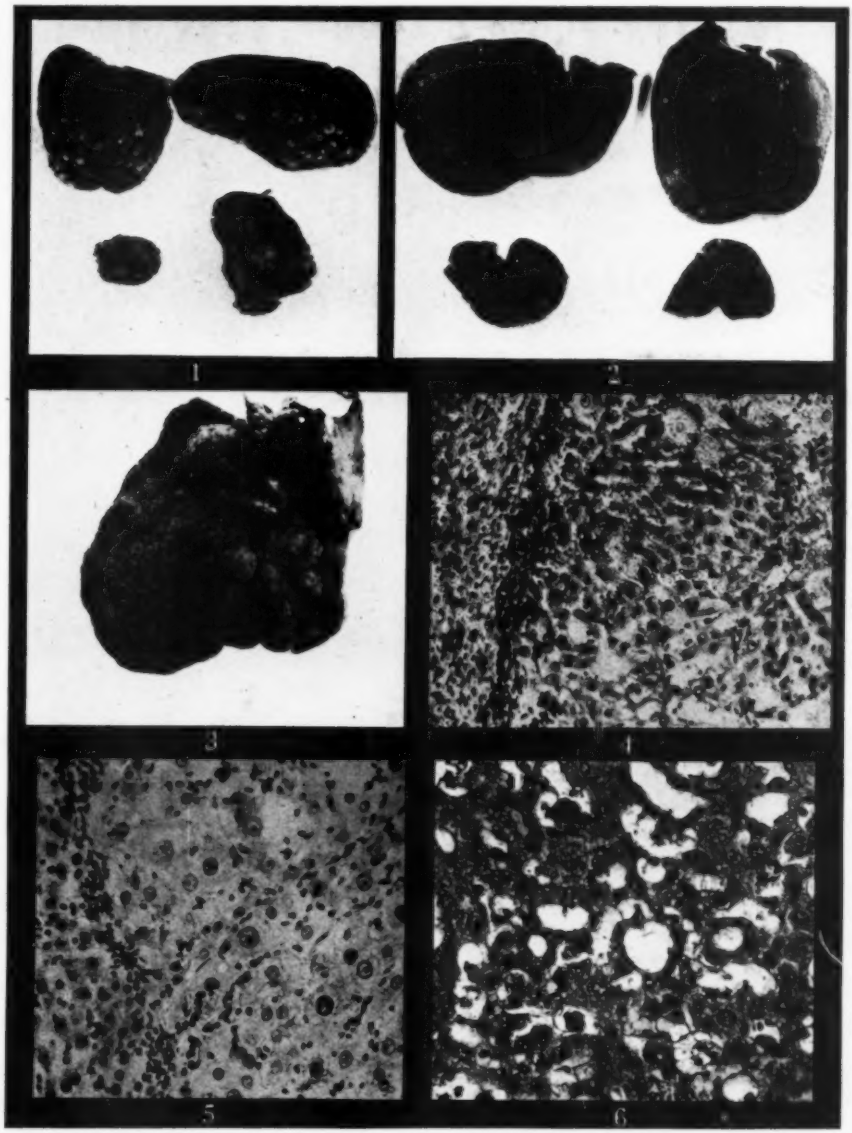
FIG. 8.—Rat 0/13 (Fig. 1). Invasion of a large blood vessel. Haematoxylin and eosin. × 215.

FIG. 9.—Rat 0/23. Total 9950 mg. tannic acid per kg. body weight, in 49 injections. Died on 294th day. Invasion of a blood vessel. Haematoxylin and eosin. × 250.

FIG. 10.—Rat T/18. Total 750 mg. tannic acid in 28 injections. Killed on 141st day. Large area of proliferated bile ducts. Haematoxylin and eosin. × 28.

FIG. 11.—Rat 0/13 (Fig. 1). Cholangeioma with incorporated hepatic cells. Haematoxylin and eosin. × 250.

FIG. 12.—Rat 0/23 (Fig. 9). Low grade adenocarcinoma. Haematoxylin and eosin. × 250.



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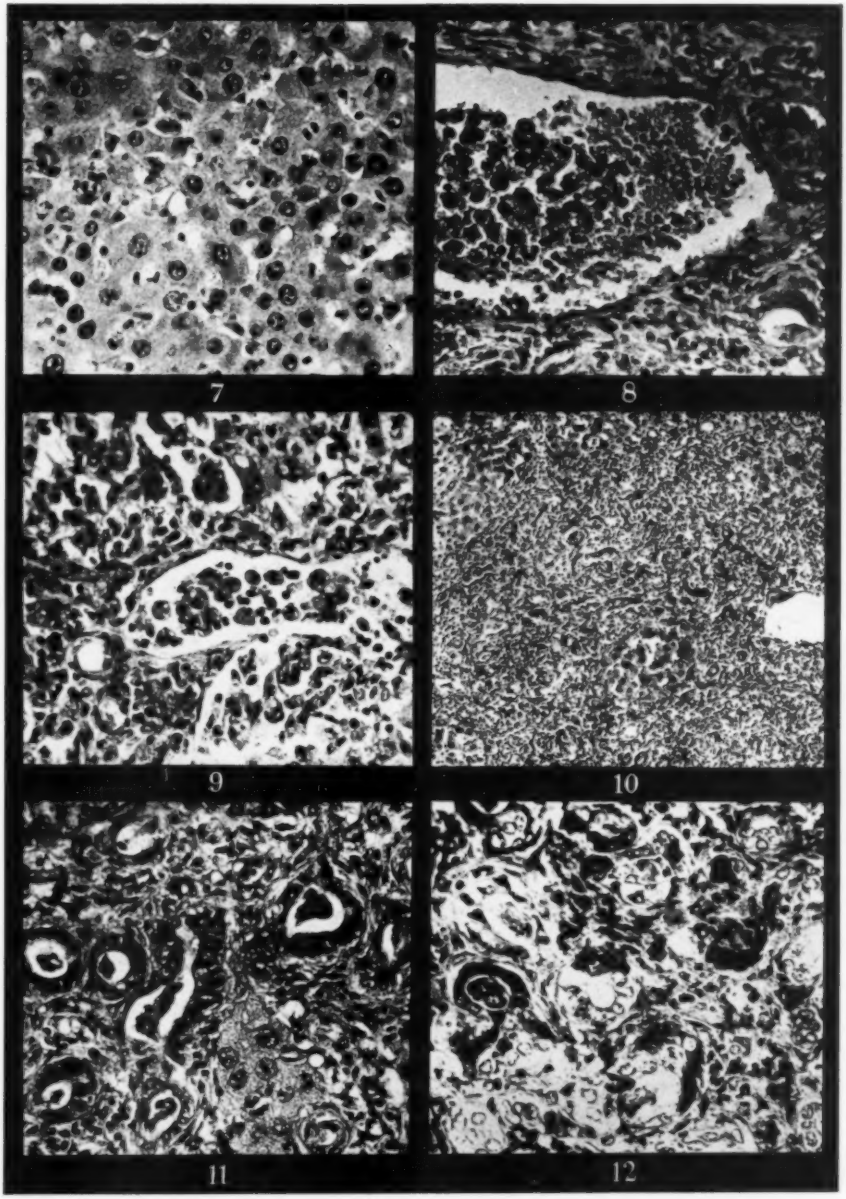

Korpássy and Mosonyi.

determined in a number of our animals with liver tumours. Whether tannic acid is the active agent in inducing hepatic tumours, or whether these tumours are merely the result of hepatic damage caused by tannic acid, awaits further study.

As tannic acid produces necrosis at the site of the injections, the question may arise whether some product of this necrosis could be responsible for the tumours. Our experiments with burn ulcers repeatedly treated locally with tannic acid or hydrochloric acid are definitely against this suggestion, for the greater part of these rats with healing-inhibited skin ulcers survived the 400th day of the treatment, and although during this time much product of necrosis could be absorbed no hepatomas or cholangeiomas arose in any of the animals.

It is noteworthy that according to Morozenskaja's (1946) investigations butter yellow is not a selective hepatic carcinogen, but can produce cancer elsewhere too. Recently Hoch-Ligeti (1949) gave an account of an experiment with rats in which no liver tumours developed after the animals had received a diet containing butter yellow for 17 months, though 3 pancreatic tumours were found occurring between the 12th and 15th months of the experiment. Our observations suggest that the tannic acid effect may be paralleled in this respect also by that of butter yellow. Although the histological examination of all the organs of the animals used in these experiments has not yet been completed, it can already be stated that bronchial adenomas were found in the lungs of some of the animals.

If tannic acid really is a carcinogenic agent, then we face a multitude of problems to be solved. Here we wish to deal quite briefly with two questions. Firstly, what is the effective agent in tannic acid? It is believed that in the organism tannic acid is split to gallic acid. Baker and Handler (1943) found, however, that gallic acid when parenterally administered was not hepatotoxic. The question now arises as to what part contamination is playing. Even purified pharmaceutical preparations of tannic acid contain, besides pentadigalloylglucose, several known or partly known organic substances in small quantities, for example, ellagic acid, quercitol and quercic acid. Tannic acid, however, seems unlikely to contain any of the carcinogenic substances known up to the present.

The second question is—can tannic acid have some part in the aetiology of human tumours? Willis (1948) writes: "There is good reason to believe that extrinsic chemical substances may play the major part in the causation of cancer of the liver." Tannic acid differs from the chemical carcinogens known so far in that it may get into the human organism, possibly in appreciable amounts, by means of fruits and various beverages, such as coffee, tea and claret. Korpássy, Koltay and Horvai (1950) from examination of the tannic acid concentration of the blood in animals found that in their experiments tannic acid administered by mouth was readily absorbed. The present authors, however, believe that the investigations hitherto made do not provide evidence of any connection with the genesis of human tumours.

SUMMARY.

Twenty-eight young albino rats have been treated with tannic acid solutions administered subcutaneously, generally every 5 days, over a long period. Ulcers were produced by burning the skin of 39 other white young rats. The ulcers in

20 animals were painted daily with 5 per cent tannic acid solution over a long period, while the ulcers of the remaining 19 animals were painted daily with hydrochloric acid.

Changes in the liver (cirrhosis, hepatomas and cholangeiomas) appeared only in animals treated with tannic acid parenterally. These induced tumours took the form of pale greyish nodules with a diameter of 2 to 8 mm. The tumours were always multiple and in general benign, although invasion of the liver veins observed in 2 cases, and an atypical pattern seen in some cases, suggest that the possibility of low grade malignancy should be considered.

Hepatic tumours appeared in 13 (56 per cent) of 23 rats which survived the 100th day of the parenteral treatment and died or were killed between the 109th and 388th days of the experiment.

Liver cirrhosis of various grades was found in 15 of the 23 rats surviving the 100th day of the parenteral treatment; in 4 of the animals with tumours no cirrhosis could be detected.

A local tumour producing effect of the tannic acid could not be demonstrated.

No great importance in tumour induction could be attached to the products of skin necrosis.

The tumour-producing and cirrhogenic effects of tannic acid are compared with the carcinogenic effects of butter yellow, *o*-aminoazotoluene, carbon tetrachloride and acetylaminofluorene.

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EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER; SCREENING TESTS WITH 6 GROUPS OF ORGANIC COMPOUNDS ON IMPLANTED MOUSE TUMOURS.

D. HAMER, I. W. HUGHES, M. STACEY, M. WEBB AND D. L. WOODHOUSE.

From the Cancer Research Laboratories, Department of Pathology, The Medical School, and the Department of Chemistry, University of Birmingham.

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UNTIL much fundamental knowledge is available concerning the mechanism of normal and neoplastic growth, efforts to discover a chemical or biological agent with selective inhibitive or lethal action upon tumour cells must be empirical in approach, and involve tests with large numbers of compounds on a variety of animal tumours. Moreover, because many variables are involved, the activity should be observed under various conditions on adequate numbers of test and control animals, so that the evaluation of chemo-therapeutic potency is a tedious procedure. Efforts have been made to overcome some of these difficulties by carrying out "screening tests" involving single or successive injections into a limited series of mice bearing grafted tumours, followed by histological examination of the tumour and organs, by tests utilizing tumour fragments transplanted to the chorio-allantoic membrane of the chick-embryo or by tissue culture techniques.

This paper describes tests on several types of grafted tumours in mice with 48 compounds belonging to 6 general chemical groups. Results were almost entirely negative, and thus indicate that these classes of compound have little therapeutic effect on implanted mouse tumours, so that more extensive trials do not seem justified with this type of compound.

EXPERIMENTAL.

Animals.

The following type of tumours were used:

Transplants from a spindle-celled sarcoma originally induced with dibenzanthracene by one of us (D.L.W.) (McDonald and Woodhouse, 1938). The tumour, which is implanted in the leg muscle tissue of albino mice, gives a high percentage of successful grafts. The new tumours are easily felt after about 7 days.

A breast tumour, originally spontaneous in a C3H mouse which has been transplanted in C3Hx (hybrid) mice through some 60 generations. The grafts develop in 100 per cent of these animals, and are palpable in about 4 to 5 days.

The "Crocker" mouse sarcoma was also used in a limited number of the tests.

Spontaneous regressions are rare in the animal strains employed once a

palpable tumour has been observed, i.e. when the inoculum has been successfully grafted. Whenever sufficient material was available the tests were done on at least two of the above types of tumour. The tumour passages and animal tests were carried out by two of the authors (D.H. and D.L.W.).

General technique.

Mice bearing small implanted tumours were injected subcutaneously or intraperitoneally with 0·2 to 0·5 ml. of solution or suspension of the chemical on alternate days and observed for regression or inhibition of growth. It is of course necessary to ensure that any effects observed on the tumour development are not due simply to toxic chemical factors affecting the health of the animal. In our experiments the dose given was usually appreciably less than the amount which produced perceptible impairment of health.

In a few cases injections were given the day following implantation of the tumours, with subsequent injections every other day. In all experiments the injected mice were compared with an equal number of control mice, approximately 10 to 12 being used for each test, which was continued for 10 to 14 days. The overall results were identical by either technique, and since these did not indicate any significant inhibition, the size and growth rates have not been detailed.

In some supplementary tests the compounds were injected intravenously in doses roughly two-fifths of those recorded below. When practicable, the compounds tested were injected in aqueous solution or, in the case of the acidic compounds, in the presence of a suitable buffer. When the substance was not sufficiently water-soluble, colloidal solutions were prepared, usually with the addition of a stabilizer, such as a small amount of gum or gelatine. Two compounds (cholyl cyanide and cholesteryl chloride, No. 6 and 15) were injected subcutaneously dissolved in cod liver oil.

Materials.

The syntheses of certain chemicals made specially for these studies are described in a later section. Most of them (No. 10, 14, 18, 22 to 29) were prepared by workers in the Department of Chemistry, University of Birmingham. The compounds (36 to 38) in Group D and all those in Group E were obtained from the Research Department of May & Baker, Ltd. (Dagenham, Essex), and made available through the co-operation of Dr. A. J. Ewins. Those in Group F were prepared in the Birmingham Medical School Cancer Research Laboratories.

Group A.

Compounds related to the sterols and bile acids.

This series (Table I) was selected from substances prepared during researches on cholic acid and its derivatives, which are of particular interest in view of their bacteriostatic properties (Stacey and Webb, 1947; Jones, Smith and Webb, 1948). The *in vitro* antibacterial action of the bile acids, particularly against certain Gram-positive micro-organisms, is well known (Sobotka, 1937). It has also been observed that certain bile salts are able to remove the ribonucleic acid component and other vital constituents from the surface of certain types of bacteria (Henry and Stacey, 1943). Components structurally related to the bile

acids were therefore submitted to *in vivo* tests on tumours to determine whether they would produce any effect on the growth of mammalian neoplastic cells.

For this study an endeavour was made to produce substances in which the original structure was altered in various ways, by the addition of new polar groups (acid or basic), or by substitution with non-polar radicals in various positions.

The compounds and dosage per injection are given in Table I. As indicated previously, in no case was any significant inhibition of tumour growth observed.

TABLE I.

	Compound.	Dosage (mg.).
1.	23-amino-3.7.12-trihydroxynorcholane	2·0
2.	23-Guanido ,, ,,	1·0
3.	24-Guanido-3.7.12-trihydroxycholane	2·0
4.	24-Amino ,, ,,	1·5
5.	Cholyl amidine hydrochloride	1·4
6.	Cholyl cyanide	0·8
7.	Cholyl amide	2·5
8.	Triformyl cholyl amide	2·0
9.	Triformyl cholic acid	0·5
10.	Triformyl cholyl diazoketone	0·6
11.	Diformyl deoxycholic acid	1·3
12.	Diacetyl deoxycholic acid	0·7
13.	3.7-Diformyl-12-ketocholanic acid	0·8
14.	Dimesyl methyl deoxycholate	0·5
15.	Cholesteryl chloride	1·5
16.	Cholyl hydrazine	1·0
17.	Cholyl ethyl urethane	0·1
18.	23-N-methyl amino-3.7.12-trihydroxynorcholane	1·0
19.	3-amino-7.12-dihydroxycholanic acid	0·3
20.	Isoamyl-3-amino-7.12-dihydroxycholanate	0·5
21.	Methyl-2-amino-7.12-dihydroxycholanate	0·5

The syntheses of the majority of the compounds listed in Table I have been recorded elsewhere, and require little further comment. The methods for the preparation for compounds 1 to 8 have been described by James, Smith, Stacey and Webb (1946), and for compounds 19 to 21 by Jones, Webb and Smith (1949). An improved method for the preparation of the formyl derivatives 8, 9, 11 and 13 has also been recorded (Hughes, Smith and Webb, 1949). References to the preparation of compounds 12, 15, 16 and 17 are summarized by Sobotka (1937).

Group B.

Amino compounds.

A considerable amount of research has been carried out on the chemotherapeutic properties of aromatic amino derivatives of several types. Boyland (1946) found that a number of diamino-diphenyl compounds inhibited growth of spontaneous tumours in mice, while other amino compounds which have given some measure of success with mouse and rat tumours include the N-substituted amino

stilbenes, and the related stilbamidines (Haddow, Harris, Kon and Roe, 1948). None of the compounds we tested (Table II) had any measurable effect on the rate of growth of the implanted tumours.

TABLE II.

	Compound.	Dosage (mg.).
22.	1.2-naphthadiamine	0·2
23.	Glucose-β-naphthanilide	0·5
24.	Glucose-α-naphthanilide	0·2
25.	N-acetyl-9-amino-anthracene	0·4
26.	Diaminophenanthrene	0·1
27.	Diaminophenenthraquinone	1·2
28.	6-aminochrysene	0·4
29.	6-12-diaminochrysene	0·2

Group C.

Halogeno compounds.

Some chlorinated hydrocarbons have been found to have strong insecticidal activity, often believed to be associated with their solubility in lipoids. Also these and other chlorinated ethanes and ethylenes have been found to affect the germination of seeds, and in particular to cause the liberation of glutathione (Guthrie, 1939).

In view of the special attributes of fluorine in organic compounds, it was considered that a short series of halogeno, saturated and unsaturated hydrocarbons, including some containing fluorine, should be tested for their capacity to inhibit tumour growth (Table III).

TABLE III.

	Compound.	Dosage (mg.).
30.	Phenyl pentachloride	5·0
31.	2.2′-Diphenyl-1.1.1-trichloroethane	0·1
32.	2.2′-Di(*p*-fluorophenyl)-1.1.1-trichloroethane	1·0
33.	4.4′-Difluorobenzophenone	0·8
34.	Tolan tetrachloride	0·2

Group D.

Compounds with redox characteristics.

Strauss, Cheronis and Strauss (1948) reported that with malignant tissues reduced tetrazolium salts to the red " formazan " form much more rapidly than normal tissues. The group of compounds listed below (Table IV) are of this type, and it was anticipated that they might have some effect on, for example, the dehydrogenase systems of the tumours. However, no inhibition whatsoever was observed with the doses given. Moreover, we were unable to confirm the original observation of Strauss, Cheronis and Strauss (1948) by tests on tumour or normal tissue slices, and Seligman, Gofstein and Rutenburg (1949) have since reported that there is no evidence of localization of such compounds in animal tumours when I^{131}-tagged compounds are used.

TABLE IV.

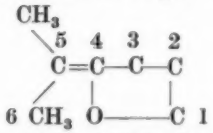
	Compound.	Dosage (mg.).
35.	2.3.5-Triphenyltetrazolium chloride . . .	0·1
36.	3.5-diphenyl-2*p*-NH_2-phenyl-tetrazolium chloride	0·1
37.	2.3.diphenyl-5*p*-NH_2- ,, ,, ,,	0·1
38.	2.5.diphenyl-3*p*-NHAc- ,, ,, ,,	0·1
39.	Resazurin	3·0

35 and 39 were ordinary reagent quality chemicals.

Group E.

Intermediates in the synthesis of penicillic acid.

The series of derivatives in Table V are intermediates obtained during studies on the synthesis of penicillic acid which all possess the basic skeleton



They were tested because of the biological (bactericidal) activity of penicillic acid and related compounds.

TABLE V.

	Compounds.	Dosage (mg.).
40.	5-Methyl-4-hydroxyhex-5-en-2-inoic acid	0·8
41.	5-Methyl-4-hydroxy-3-methoxyhexa-2:5 dienoic acid lactone . .	1·0
42.	5-Methyl-4-hydroxy-3-methoxyhexa-2:4 dienoic acid lactone . .	0·4
43.	6-Bromo-5-Methyl-4-hydroxy-3-methoxyhexa-2:4-dienoic acid lactone	0·4
44.	5-Methyl-5-hydroxy-3-methoxy-4-ketohex-2 enoic acid . . .	5·0

Group F.

Zinc derivatives.

Evidence has been found of significant differences between the zinc content of normal and some malignant tissues, the latter often having a much higher content (Heath, 1949).

Furthermore, various amino acids (Beard, 1942) and malonic acid have been found to have inhibitory effects on tumour growth (Boyland, 1940; Woodhouse, 1947). The inhibition in the form of such co-ordinated metallic compounds was thought to offer some advantage. The compounds showed moderate toxic properties ; the doses shown in Table VI are close to the maximum tolerated by animals of 25 to 30 g. weight given repeated injections.

TABLE VI.

	Compounds.	% Zinc.	Dosage (mg.).
45.	Zinc-glycine . .	15·5	1·0
46.	Zinc-tyrosine . .	10·1	1·3
47.	Zinc-glutamic acid . .	20·4	0·9
48.	Zinc-Malonic acid . .	31·5	1·0

The compounds were prepared by warming the amino acid in the presence of excess freshly precipitated zinc hydroxide. After filtering off excess zinc hydroxide the compounds were isolated by careful evaporation and crystallization. The zinc content was determined on the dried preparations by Lang's ferricyanide-iodide method.

Special Syntheses.

Triformyl cholyl diazoketone (10), isolated as an intermediary in the synthesis of homocholic acid, was prepared from triformyl cholyl chloride and diazomethane according to the method of Ruzicka, Plattner and Heusser (1944). Repeated recrystallization from Grignard-dried methanol gave triformyl cholyl diazoketone as white needles (m.p. 135 to 136°, $[\alpha]_D^{20} + 86{\cdot}2$, in chloroform (c = 1·1). (Found: C, 64·9; H, 7·6.; Calc. for $C_{28}H_{40}O_7N_2$; C, 65·1; H, 7·7 per cent.)

Dimesyl methyl deoxycholate (14).—Methyl deoxycholate (2 g.) was thoroughly dried by distillation with sodium-dry ethanol and benzene, and the resulting syrup dissolved in dry pyridine (4 c.c.). The solution was cooled in ice, and freshly distilled methane sulphonyl chloride (1 c.c.) added in 5 equal proportions of 0·2 c.c., over a period of approximately 1 hour. After standing at room temperature for 3 days, a few drops of water were stirred into the dark nearly-solid mixture, and the whole stirred into excess water. The sticky solid which separated hardened on trituration, and was filtered off. The aqueous filtrate was extracted with chloroform (6 times) and the extract washed with water, ice-cold 1N hydrochloric acid (5 times) to remove pyridine, again with water, sodium bicarbonate solution, and finally with water. After drying ($CaCl_2$), evaporation of this extract yielded only a very small amount of a brown syrup, indicating that only a small fraction of the product remained in aqueous solution.

The filtered solid was dissolved in ethanol and a little acetone, and the solution slowly run into water (800 c.c.) with vigorous stirring. The brownish solid which separated was filtered off and dried. It could not be crystallized from any of the various solvents used. The product was therefore dried as before by distillation with dry benzene and ethanol, dissolved in dry pyridine and again treated with mesyl chloride (1 c.c.). After standing overnight at room temperature the solution was heated at 50° for 1 hour, and then worked up as before. An initial purification was effected by dissolving the crude product in acetone and stirring into brine. The precipitated solid was dissolved in acetone and the solution decolorized with charcoal. Evaporation of the solution gave a syrup which crystallized in colourless monoclinic plates from a mixture of dry acetone, dry benzene and dry light petroleum. The crystals (1·05 g., 38 per cent) had m.p. 63°, depressed to 57° in admixtures with methyl deoxycholate (m.p. 63·50), and showed $[\alpha]_D^{12} + 50°$ in chloroform (*c*, 1·0). (Found: S, 11·8; Calc. for $C_{27}H_{46}O_8S_2$; S, 11·4 per cent.)

23-*N-methylamino*-3:7:12-*trihydroxynorcholane* (18).—An attempt was made to increase the basicity of 23-amino-3:7:12-trihydroxynorcholane by the formation of its N-methyl derivative following the method of Eckhardt (1938) for the preparation of N-methyl-7-amino-cholestane. The first step in the reaction involved the preparation of the free base from the hydrochloride. Neutralization of an ethanolic solution of the latter with sodium hydroxide in ethanol proved unsuitable, since water liberated during the reaction, as well as traces of carbonate in

the alkali, tended to react with the free base. The following method of synthesis proved suitable:

A solution of 23-amino-3:7:12-trihydroxynorcholane-hydrochloride (2 g.) in ethanol (10 ml.) containing sodium ethoxide (from 0·11 g. Na) was kept at 60° for 2 hours. The cooled solution was filtered to remove the precipitate of sodium chloride, and evaporated to dryness under diminished pressure at 50° (bath temperature) in an atmosphere of nitrogen. The dry residue was extracted (3 times) under reflux with dry ether (10 ml.). The combined ethereal extracts (in nitrogen) were cooled in ice and methyl iodide (3·5 ml.) added. The white crystalline product which separated on standing overnight at 0° was collected and dried *in vacuo* at room temperature. Recrystallization from acetone containing hydriodic acid (0·02N) gave N-methyl-23-amino-3:7:12-trihydroxynorcholane hydroiodide (0·8 g., m.p. 268 to 269°). Found: C, 54·9; H, 2; N, 2·4. $C_{24}H_{44}O_3NI$ requires C, 55·1; H, 8·4; N, 2·6 per cent.)

1:2 *Naphthadiamine* (22).—Treatment of 2-naphthylamine (10 g.) in warm ethanol (65 ml.) with a solution of benzene diazonium chloride from aniline (7 g.) according to the method of Bamberger and Schieffelin (1889) gave 1-Benz-azo-2-naphthylamine (10·2 g., m.p. 102–104°, after recrystallization from ethyl acetate). The latter compound (5 g.) was reduced with zinc dust and acetic acid in the usual way, and the resulting solution poured into 0·2 *N* sulphuric acid (100 ml.). The precipitated sulphate was dissolved in warm 5*N* sodium hydroxide (40 ml.) and the solution filtered. On cooling, light brown crystals separated, which were filtered, washed with water and dried. Recrystallization from aqueous ethanol yielded needles of 1:2-diaminonaphthalene (1·2 g., m.p. 97°).

Glucose-β-naphthadiamine (23).—A solution of 2-naphthylamine (2 g. purified by sublimation at 112°) in ethanol (30 ml.) was boiled under reflux with glucose (1 g.) for 1 hour. Excess ethanol was then removed by evaporation, and the residual solution treated with ether (80 to 100 ml.). The crystals of glucose-β-naphthanilide (0·8 g., m.p. 115 to 117°) which separated on standing were filtered, washed with ether and dried *in vacuo*.

Glucose- -napththanilide (24) was prepared from 1-naphthylamine (purified by sublimation at 50°) and glucose in an identical manner.

N-acetyl-9-aminoanthracene (25).—9-Aminoanthracene was prepared from anthracene by the formation and subsequent reduction of 9-nitroanthracene according to the method of Dimroth (1901). The N-acetyl derivative was formed when a solution of 9-aminoanthracene (1·2 g.) in redistilled acetic anhydride (7 ml.) was allowed to stand in the cold. The crystalline product which separated was collected, washed first with acetic acid and then with ethanol and dried. Recrystallization from aqueous ethanol yielded N-acetyl-9-aminoanthracene (0·8 g., m.p. 273 to 274°).

9:10-*Diaminophenanthrene* (26) was obtained by the reduction of phena-n thraquinone dioxine in ethanol solution with zinc chloride-hydrochloric acid according to the method of Schmidt and Soll (1907). The crude product was recrystallized from ethanol to give 9:10 diamino phenanthrene (1·1 g., m.p. 154 to 155°) as needles. (Found: N, 16·4; Calc. for $C_{14}H_{12}N_2$; N, 16·6 per cent.)

2:7-*Diaminophenanthraquinone* (27).—A solution of phenanthraquinone (10 g.) and methylamine hydrochloride (8·8 g.) in dry benzene (35 ml.) was boiled under reflux for 6 hours (Jaffe and Day, 1943). The residual solid was extracted repeatedly with boiling benzene (50 ml.). The combined benzene extracts were

filtered to remove unchanged phenanthraquinone (2·1 g., m.p. 185 to 195°), and evaporated under reduced pressure at 50° (bath temperature). The residual yellow solid was extracted with 5N hydrochloric acid, ethanol, and then with ethylacetate. Evaporation of the ethyl acetate extract to half-volume under reduced pressure gave a solid which, on crystallization and recrystallization from ethanol, afforded 2:7-diaminophenanthraquinone (1·2 g., m.p. 209 to 210°).

6-*Amino-chrysene* (28) was prepared by the following modification of the method of Newman and Cathcarte (1940). Reduction of the intermediary, 6-nitro-chrysene was more efficiently carried out with iron filings and hydrochloric acid than with red phosphorus and hydriodic acid.

To a solution of chrysene (10 g.) in glacial acetic acid (280 ml.) was added a mixture of concentrated nitric acid (8 ml.) and concentrated sulphuric acid (10 ml.) in glacial acetic acid (100 ml.). The yellow precipitate which separated from the reaction mixture was collected after 30 minutes, dried and recrystallized from aqueous pyridine. The resulting product was dissolved in absolute benzene, and the solution passed first through a column of alumina and then through silicon dioxide. Evaporation of the benzene solution gave 6-nitro chrysene (7·5 g, m.p. 212 to 213°).

Reduction of 6-*nitrochrysene.*

(a) *With red phosphorus and hydriodic acid.*—A mixture of 6-nitrochrysene (2 g.) and red phosphorus (0·5 g.) in hydriodic acid (D 1·7, 4·5 ml.) was heated under reflux on a sand tray and glacial acetic acid (10 ml.) cautiously added. After 1 hour the mixture was cooled, and the reddish-brown solid which separated extracted several times with benzene (60 ml.). The combined benzene extracts were washed with water, sodium thiosulphate solution and again with water. After drying ($MgSO_4$), the solution was concentrated under diminished pressure in an atmosphere of nitrogen at 40° (bath temperature) to yield a white solid (m.p. 270 to 276°). The latter was rapidly filtered and dissolved immediately in acetic anhydride (8 ml.) and glacial acetic acid (3 ml.). After 30 minutes at room temperature the solution was poured into water, and the white precipitate which separated collected, washed with water and dried. Recrystallization from aqueous ethanol afforded 6-N-acetyl-aminochrysene (0·37 g., m.p. 297 to 299°).

(b) *Iron filings and hydrochloric acid.*—6-nitrochrysene (4 g.) was added with vigorous stirring to a mixture of iron filings (2·1g.), 5 N-hydrochloric acid (20 ml.), and water (5 ml.) at 95 to 100°; when the 6-nitrochrysene had dissolved completely the heating was discontinued, and sodium carbonate (6 g.) added. The solution was then cooled, filtered and evaporated under reduced pressure. The residue was extracted with ethanol, the combined extracts dried and evaporated under reduced pressure at 40°. The crystalline product which separated was recrystallized from ethanol to give 6-aminochrysene (2·7 g., m.p. 274 to 275°).

6:12-*Diaminochrysene* (29).—Chrysene (6 g.) in glacial acetic acid (210 ml.) was treated at 90° with concentrated nitric acid (63 ml.) and concentrated sulphuric acid (6 ml.) to give 6:12-dinitrochrysene (7·3 g., m.p. 372 to 376°). The latter (5 g.) was added slowly with vigorous stirring to a mixture of 5*N* hydrochloric acid (25 ml.) and iron filings (4·2 g.) at 95°. After 2 hours sodium carbonate (5 g.) was added, the solution cooled and filtered. The filtrate was extracted several times with ether (100 ml.), the combined ethereal extracts washed with

water, dried ($MgSO_4$), and evaporated to dryness. The residual white solid, on recrystallization from ethanol, gave 6:12 diaminochrysene (3·9 g.), which was converted to its stable diacetyl derivative in the usual way. Recrystallized from aqueous ethanol, the 6:12-diacetylamino chrysene formed needles (362 to 365°).

SUMMARY.

Forty-eight chemicals belonging to 6 general groups have been tested for tumour inhibition by injection into mice bearing grafted sarcomas and carcinomas with negative results.

The groupings were :

A. Compounds related to the sterols and bile acids.
B. Amino compounds.
C. Halogeno-compounds.
D. Compounds with Redox characteristics.
E. Intermediates in the synthesis of penicillic acid.
F. Zinc derivatives of amino acids.

The syntheses of the compounds specially prepared for this work are described.

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EFFECT OF *P*-DIMETHYLAMINOAZOBENZENE, *O*-AMINOAZOTOLUENE, BENZPYRENE AND 1:2:5:6-DIBENZANTHRACENE ON NICOTINIC ACID SYNTHESIS IN LIVER TISSUE.

H. N. De and S. R. GUHA.

From the Biochemistry and Nutrition Laboratory. Dacca University, Dacca, Pakistan.

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In recent years no subject has received so much attention as tumours, but the complexities regarding the action of the different factors responsible for such neoplastic growths have not yet been fully elucidated. Of the different accessory factors studied, the vitamins seem to play a predominant role in tumour growth, and by recent researches it has been revealed that there exists a relationship between the dietary vitamins on the one hand and the vitamin contents of the tumour on the other. Vitamins have been found to act both carcinogenically and anticarcinogenically, depending on the nature of the tumour and of the carcinogen used. In this line of vitamin-cancer relationship a large amount of work has been done with hepatomas produced by feeding the azo dye, *p*-dimethylaminoazobenzene, and it has been observed that in the case of such tumours nicotinic acid and riboflavin when administered to the animals manifest anticarcinogenic activity (Gyorgy, Poling and Goldblatt, 1941; Kensler, Sugiura, Young, Halter and Rhoads, 1941), whereas the other members of B-complex, as pyridoxine (Miner, Miller, Baumann and Rusch, 1943; Miller, Baumann and Rusch, 1945), inositol (Miller, Miner, Rusch and Baumann, 1941; Burk, Kensler, Rhoads, Sugiura and du Vigneaud, 1942), biotin (du Vigneaud, Spangler, Burk, Kensler, Sugiura and Rhoads, 1942; Burk, Spangler, du Vigneaud, Kensler, Sugiura and Rhoads, 1943) and *p*-aminobenzoic acid (Burk, Kensler, Rhoads, Sugiura and du Vigneaud, 1942) acted procarcinogenically. Studies on the vitamin contents and the enzyme activity of hepatomas and normal liver have revealed that the contents of nicotinic acid and riboflavin and of the enzymes constituted by these vitamins are lower in the case of hepatomas than in liver, whereas those of pantothenic acid, thiamine, pyridoxine and biotin remained almost unchanged (Burk and Winzler, 1944). The causes of the above diverse effects of the different members of B vitamins on the development of hepatomas and of the variations in their concentrations in such tumours have not yet been fully elucidated, and the present investigation is an attempt to achieve this object.

Recent investigation from this laboratory by the authors (De and Guha, 1950) has revealed that rat liver tissue can convert tryptophane to nicotinic acid when incubated with it. This prompted us to investigate whether the lowered values of nicotinic acid and coenzyme I in the hepatoma as observed by different workers is due to interference with the above synthetic capacity

of liver in producing nicotinic acid by the carcinogen, *p*-dimethylaminoazobenzene. Other carcinogens as *o*-aminoazotoluene, benzpyrene and 1:2:5:6-dibenzanthracene were also found to produce tumours and effect a change in the different vitamin contents in the liver (Carruthers, 1942; Sasaki and Yoshida, 1935; Baumann, Foster and Lavik, 1941), and the studies of the effect of these carcinogens on the above synthetic process of the liver has also been dealt with in the present investigation.

EXPERIMENTAL.

Normal adult rats were killed by a blow on the head. The liver was immediately removed and thin slices were prepared and kept immersed in Ringer-Locke solution. These tissue slices were then incubated with the solution of the substrate tryptophane (precursor of nicotinic acid) with and without any of the four carcinogens, *p*-dimethylaminoazobenzene, *o*-aminoazotoluene, benzpyrene and 1:2:5:6-dibenzanthracene. The whole experiment was divided into 3 sections as detailed below :

(*a*) Control experiment with a set of six 50 c.c. Erlenmeyer's flasks, to each of which were added 5 c.c. of buffered Ringer-Locke solution (prepared by adding 10 c.c. of pH 7·4 phosphate buffer to 100 c.c. of Ringer-Locke solution), 10 c.c. of distilled water and a few slices of liver tissue.

(*b*) Substrate-experiment with another set of six conical flasks, to each of which were added 5 c.c. buffered Ringer-Locke solution, 10 c.c. of the substrate solution containing 1000 μg. of L-tryptophane, and about the same number of tissue slices as were used in the control experiment.

(*c*) Experiment with substrate plus carcinogen with another set of flasks, to each of which were added equal quantities of the substances used in section (*b*) above, and an additional 5 c.c. of 0·3 per cent benzene solution containing 100 μg. of any of the above four carcinogens.

To each of the flasks of the control and substrate experiments were added 5 c.c. of 0·3 per cent benzene solution without any carcinogen to make the experimental conditions identical.

All the above steps were completed within the least possible period, and the flasks were then kept at a constant temperature of 37° C. for 4 hours and stirred well at regular intervals.

The wet weights of 6 batches of equal numbers of tissue slices as used in the above 3 sets of experiments were taken separately, and from their average values the wet weights of the tissue slices actually used for incubation were assessed.

After completion of the incubation period the contents of the flasks with the tissue slices were homogenized in a Waring blender, and their total nicotinic acid contents were estimated according to the method adopted by Swaminathan (1942), with some modifications according to Wang and Kodicek (1943). The values estimated by these methods represent free nicotinic acid, nicotinuric acid, nicotinamide and also trigonelliene in which N′-methylnicotinamide is also included, and all these have been expressed as total nicotinic acid per gramme of wet tissue.

The results presented in Table I show that a large amount of L-tryptophane is converted to nicotinic acid when incubated with liver tissue slices, but that

this synthesis is inhibited to an appreciable extent when any of the 4 carcinogens, *p*-dimethylaminoazobenzene, *o*-aminoazotoluene, benzpyrene or 1:2:5:6-dibenzanthracene, is added along with L-tryptophane in the culture medium. The results indicate that the low nicotinic acid and coenzyme I contents of hepatomas

TABLE I.—*Showing the Effect of* p-*Dimethylaminoazobenzene,* o-*Aminoazotoluene, Benzpyrene and* 1:2:5:6-*Dibenzanthracene on the Conversion of* L-*Tryptophane to Nicotinic Acid by Rat Liver Tissue. The figures represent the average values for* 6 *culture experiments.*

Substrate and carcinogen used.	Wet weight of tissue. (g.).	Nicotinic acid content in μg. per g. of wet tissue.
Nil (Control)	0·26	226·8
L-Tryptophane (Substrate) . . .	0·31	478·4
L-Tryptophane + *p*-dimethylaminoazobenzene . .	0·24	244·7
L-Tryptophane + *o*-aminoazotoluene . .	0·30	265·9
L-Tryptophane + benzpyrene . . .	0·25	310·4
L-Tryptophane + 1:2:5:6-dibenzanthracene . . .	0·28	265·0

induced by feeding *p*-dimethylaminoazobenzene to rats as observed by Pollack, Taylor and Williams (1942), Taylor, Pollack, Hofer and Williams (1942) and Kensler, Sugiura and Rhoads (1940) might be due to interference with the above synthetic capacity of liver tissue to produce nicotinic acid under the influence of the above carcinogen. Similar explanations may be offered for the other three carcinogens used in the present investigation.

In the recent investigation from this laboratory by the authors (De and Guha, unpublished data), it has been shown that some enzyme systems, such as are involved in the conversion of tryptophane to nicotinic acid in the liver tissue, probably contain the -SH sulfhydryl group, and it may therefore be conceived that the observed inhibition in the conversion of tryptophane to nicotinic acid by the above carcinogens is possibly due to blocking of the above -SH groups by these carcinogens or by their split products. The observation of Greenstein (1942) that rat hepatomas contained less free -SH group-containing amino acid corresponding to glutathione than rat liver, and of Potter (1942), who contends that the oxidized derivatives of *p*-dimethylaminoazobenzene inhibited the action of -SH group-containing enzymes as urease and succinic oxidase systems, lend strong support to the above concept.

Although it has not yet been definitely established that tryptophane is the true precursor of nicotinic acid in the liver, it may be conceived that, whatever be the endogenous nitrogenous precursor of nicotinic acid synthesis in the liver, the chief action of the above carcinogens is probably due to their interference with some enzyme systems containing the -SH group which might be involved in the above process of synthesis. The possibility of the involvment of the transaminase or carboxylase systems or both together in the above process of nicotinic acid synthesis from the endogenous nitrogenous source in the liver, and their inhibition by the above carcinogens or by their split products, cannot be ruled out if judged in the light of the observations of Cohen, Hekhuis

and Sober (1942), and Kensler, Young and Rhoads (1942), that the split products of *p*-dimethylaminoazobenzene inhibit the actions of transaminase and carboxylase.

SUMMARY.

The addition of carcinogens such as *p*-dimethylaminoazobenzene, *o*-amino-azotoluene, benzpyrene and 1:2:5:6-dibenzanthracene to the culture medium of liver slices incubated with tryptophane showed that the conversion of this amino acid to nicotinic acid by the liver tissue was markedly decreased under the influence of these carcinogens.

The inhibition was postulated as probably due to the interference of these carcinogens or their split products with some -SH group-containing enzyme systems which are probably involved in the process of the conversion of tryptophane to nicotinic acid in the liver, as seemed likely from the previous investigation from this laboratory (De and Guha, unpublished data).

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